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COMPUTER-AIDED INVESTIGATION OF THE
STAPHYLOCOCCUS-MICROCOCCUS COMPLEX

by



MANJIT SIHOTA

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

DEPARTMENT OF BACTERIOLOGY

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UNIVERSITY OF ALBERTA
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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled COMPUTER-AIDED INVESTIGATION OF THE STAPHYLOCOCCUS-MICROCOCCUS COMPLEX submitted by Manjit Sihota in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

Sixty-two strains of Staphylococci and Micrococci were studied. Gram negative bacilli (62 strains) were used for comparison purposes. All strains designated as Staphylococcus were positive in conversion of nitrate to nitrite, casein hydrolysis, lipolysis, hippurate hydrolysis, mannitol fermentation and M.R. test. All produced catalase and were tolerant to 10% sodium chloride and 0.02% sodium azide. All but one strain of Staph. aureus produced DNase, coagulase and phosphatase. None of the Staph. albus strains produced these three enzymes in quantities detectable by the methods used.

The results of biochemical analyses of Micrococci showed a large number of combinations of characters. With the exception of M. halophilus, none of the strains produced penicillinase. All the strains except M. freudenreichii NCIB 2699 produced catalase.

Two patterns were observed on visual spectroscopic study of the cytochromes. The organisms designated Staphylococcus had cyt b and cyt a. Most of the organisms designated Micrococcus had cyt b, cyt c, cyt a pattern. A few of the Micrococci, M. halophilus, M. tetragenus, M. candidus NCIB 8610, M. aurantiacus NCIB 8609, M. cyaneus NCIB 9148, M. cyaneus CCM 856, and M. freudenreichii NCIB 2699, were characterized by a staphylococcal pattern of cytochromes and also resembled the staphylococcal group in some of the biochemical tests. A high degree of correlation was found between the oxidase test, utilization of glucose, and cytochrome pattern. Organisms with cyt b, cyt c, cyt a, were oxidase positive and attacked glucose oxidatively. Organisms designated

Staphylococcus and a few of the organisms designated Micrococcus had cyt b, cyt a, were oxidase negative and attacked glucose oxidatively and fermentatively. In the study of 62 Gram positive and 52 Gram negative organisms, the only exceptions were M. cyaneus NCIB 9148, M. cyaneus CCM 856, and M. aurantiacus NCIB 8609, which had Staphylococcus-like cytochrome pattern, were oxidase negative, but did not attack glucose fermentatively.

Analysis of biochemical data and cytochrome patterns was done with the computer program, which makes use of a measure of similarity for clustering the organisms. The same technique was applied to the gas chromatograms of whole cells grown on NYA and NYB media and their cell components (nucleic acids and protoplasm). The results showed the limited use of nucleic acids and protoplasm, but on whole cell analysis the organisms were arranged mainly in two types of cluster, one of 'typical' Micrococci and the other 'typical' Staphylococci. Some of the organisms designated Micrococcus which resemble Staphylococci in some respects and Micrococci in others were consistently clustered with Staphylococci on separate computer analysis of their biochemical tests and cytochrome patterns and gas chromatographic data. The strains of M. cyaneus NCIB 9148, M. cyaneus CCM 856, and M. aurantiacus NCIB 8609 were not placed in any of the clusters derived from biochemical tests, and M. roseus NCIB 8175 was placed with Staphylococci on the basis of its chromatogram.

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LIST OF ABBREVIATIONS

APL	- A Programming Language
cyt a	- cytochrome 'a'
cyt b	- cytochrome 'b'
cyt c	- cytochrome 'c'
DNase	- deoxyribonuclease
Fig.	- Figure
MR	- methyl red
NYA	- nutrient yeast agar
NYB	- nutrient yeast broth
O/F	- oxidation/fermentation
UV	- ultra violet
V-P	- Voges-Proskauer
V/V	- volume by volume
% W/V	- percent weight by volume

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I

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

Classification within the Staphylococcus and Micrococcus complex has been an enigma to the bacterial taxonomist. It is one of the most confusing examples of taxonomy in microbiology. The close resemblance of the organisms in their morphological, physiological and cultural characteristics has made it difficult to separate them into meaningful subgroups. Approaches to the classification of these organisms range from consideration of criteria such as pigment production and pathogenicity, to a few complex series of biochemical tests which were selected at random. Pigment production and pathogenicity, which seem to depend on so many external factors which are difficult to control, have been regarded as unreliable. For example, the production of pigment varies with the type of medium, the amount of moisture available, the amount of oxygen, and the age of the culture. In the same way, organisms usually regarded as non-pathogenic may at times produce infection in debilitated patients or in those who lack normal immunological mechanisms because of genetic defects or immunosuppressive therapy. Biochemical tests selected at random have made a considerable contribution to classification. Firstly, they have provided some information regarding the physiological and biochemical nature of the organisms. Secondly, on the basis of the data now available in the literature, it is possible to make a selection of tests which have proved to be of taxonomic value and hence could be used in the future to identify an unknown organism or, in combination with newer approaches,

to improve the present classification.

The classification of Gram positive cocci commonly designated as Staphylococci and Micrococci has not reached a stage which is entirely satisfactory. There are discrepancies in the methods used, and some of the biochemical tests which are considered valid for classification require better standardization.

On the basis of reports in literature and preliminary work carried out for this thesis, 31 characteristics, including cytochrome patterns, were selected as being probably suitable for the classification of the organisms in this complex. In addition, gas chromatograms which seemed to have potentialities for taxonomic studies were investigated.

A numerical taxonomic method has been used to interpret the results obtained from the above procedures. It was hoped that by this approach the taxonomic position of certain strains would be to some extent clarified, and that the validity and potentialities of pyrolysis gas chromatography and visual spectroscopy in this field could be assessed.

LITERATURE REVIEW

Staphylococci and Micrococci are Gram positive organisms which show close similarities in colonial and microscopic morphology and show slight differences in biochemical reactions. Many classifications of these organisms have been suggested. Some authors place them in a single group or genus and others into two or more genera. There is no doubt that the classification and nomenclature of this group of organisms is one of the most confusing examples of taxonomy in microbiology. In order to introduce current opinions concerning the classification of this group, it is pertinent to review some of the earlier attempts to define and establish the genera Staphylococcus and Micrococcus.

The foundation for the current classification of bacteria was laid down by Ehrenberg (16) nearly a century ago. He divided his vibriona, which corresponded to the bacteria, into five genera; Bacterium (straight rigid filaments); Vibrio (straight filaments showing serpentine motion); Spirillum (rigid spiral filaments); Spirochaeta (flexible spiral filaments); and Spirodiscus (spirals flattened like discs). However, no attempt was made to classify the spherical forms with other bacteria. Hallier (16) used the word 'Micrococcus' in a general sense to designate a growth form of a mold, in accordance with the theory of pleomorphism. The same name was used by Cohn (17) in a generic sense for very small, spherical or oval organisms with colorless or faintly colored cells. The organism to which this name was given was Micrococcus prodigiosus. This organism is a rod, and the next organism

described was Micrococcus luteus which is considered as the type species of this genus. The name Staphylococcus was originally proposed by Ogston (17) in a general sense for spherical organisms observed by him in human pus. In the generic sense this name was first used by Rosenbach (17), who was able to grow and isolate these organisms in pure cultures. Finally Zopf (16) established a distinct group for the spherical bacteria and put them in the family Coccaceae. In 1890 Flugge (6) placed the Staphylococci into the Coccaceae family. Further subdivision of Coccaceae has been an enigma for the bacterial taxonomists. It has gone from the simple channels of classification, such as pigment formation, to the most complicated network of physiological, biochemical, and numerical analysis.

Pigment formation as a basis of early classification

Rosenbach (17) divided Staphylococci on the basis of pigment formation. He named the orange pigmented cocci as Staphylococcus pyogenes aureus and the white pigmented cocci as Staphylococcus pyogenes albus. Later he adopted the binomials Staphylococcus aureus and Staphylococcus albus. In 1900 Migula (17) divided the Coccaceae into five genera: Streptococcus, Micrococcus, Sarcina, Planococcus and Planosarcina. Micrococcus was subdivided by pigment: white (101 species, including Micrococcus pyogenes); yellow (55 species, including Micrococcus aureus); and red (23 species); blue and violet (3 species). More than any other workers, Winslow and Rogers (73) gave most weight to pigmentation and divided the Coccaceae into two sub-families, the

Metacoccaceae and the Paracoccaceae. The orange pigmented cocci were placed by Winslow and Winslow (6) in the genus Aurococcus and the white pigmented cocci in the genus Albococcus and both were placed with Streptococci in the Paracoccaceae. The micrococci were placed with Sarcina and Rhodococcus in the Metacoccaceae. Winslow et al. (74) later re-studied the genera Aurococcus and Albococcus and concluded that both were strains of Staphylococcus aureus which had lost some of the traits of this species through growth under unfavorable conditions. They also produced an elaborate tinctorial chart by which the colors could be graded. Although Kligler (43) accepted the arrangement, it was stated by Walker and Adkinson (18), Julianelle (39) and Fairbrother (24) that pigment alone was insufficient for differentiation, as the formation of pigment depends upon the growth conditions and the type of media used. For example, the pigment production will vary with the amount of oxygen, the amount of moisture available, and the age of the culture.

Since no classification is complete without nomenclature, attempts were made to re-name the sub-families of Coccaceae on the basis of the International Rules for Botanical Nomenclature. Buchanan (12) showed that the sub-family's names Metacoccaceae and Paracoccaceae were invalid and proposed that they should be replaced by the tribal names Micrococcaceae Trevisan and Streptococceae Trevisan respectively. His suggestion was based on article 23 of the International Rules for Botanical Nomenclature, which states that: "Names of subfamilies are taken from the name of one

of the genera in the group, with the ending 'oideae'". The subfamily name Paracoccaceae did not conform to either of these requirements. No genus Paracoccus was ever described. The most characteristic and most commonly recognized genus belonging to this group was the Streptococcus. A suitable subfamily name would therefore be Streptococcoideae. A subfamily is a group interpolated between the group family and tribe, and Buchanan thought that it was appropriate to reduce the subfamily to a tribe wherever such additional grouping was desired. However, according to article 23 of the Code, the tribe should be named after one of the constituent genera with the ending 'eae'. Therefore strict adherence to the Rules of Nomenclature necessitated the substitution of Paracoccaceae by Streptococcaceae Trevisan and Metacoccaceae by the tribe Micrococcaceae. This change in nomenclature was accepted by the 1917 (15) and 1920 (16) Committees of the American Society of Bacteriology on Classification of Bacteria. Although Staphylococci and Micrococci were clearly separated, agreement was divided at this time on the validity of separation, as pigmentation was found to be an unreliable method of classification, and biochemical and physiological methods were not at that time readily accepted.

Biochemical tests as a basis of classification.

Cohn (16) was the first to make use of physiological activities of the organism as a basis for classification. He found Micrococci difficult to classify on the basis of the shape and the size of the cells. He arranged them into three groups: the chromogenic, zymogenic and

pathogenic cocci. The chromogenic cocci were divided into two sub-groups. The one with the insoluble pigment included Micrococcus prodigiosus and Micrococcus luteus, and the other with the soluble pigment included Micrococcus aurantiacus, Micrococcus chlorinus, Micrococcus cyaneus, and Micrococcus violaceus. Zymogenic cocci were represented by Micrococcus ureae, which according to Pasteur and others, formed chains and broke down urea to ammonium carbonate. The pyogenic cocci included Micrococcus vaccineae, the small cocci seen in fresh lymph, Micrococcus septicus, found in wound secretions and in tartar from decayed teeth.

At the time when Winslow and other workers were trying to classify cocci by pigment Andrews and Gordon (18) were attempting to correlate pigmentation and certain biochemical reactions with pathogenicity. They divided the Staphylococci into four groups and found that the white variants of golden strains were similar to the parent strains in gelatin liquefaction, nitrate reduction, and production of acid from maltose, lactose, glycerol and mannitol. This work clearly showed that pigmentation was not a reasonable criterion to be used for the classification of Staphylococci. On the other hand, biochemical classification also caused a great deal of confusion. Cummins and Cummings (18) found that sugar reactions were not suitable, as many of their strains changed in fermentative power or at least gave different test results when re-tested two or three months later.

Up to this point, most of the focus was on the classification

of Staphylococci but in the 1920's Hucker (34, 35, 36, 37) deviated from this and published a series of monographs on the whole group, i.e. Micrococcus and Staphylococcus. He put all the strains labelled Micrococcus, Staphylococcus, and Rhodococcus under Micrococcus. Later, on the basis of pigmentation, nitrate reduction, use of ammonium phosphate and urea as nitrogen sources, gelatin liquefaction and mass or tetrad formation, he divided the genus Micrococcus into 16 species. Cowan (18) has stated that Hucker's ideas have formed the basis of the present classification of Micrococci, as most of the strains with which he was working were Micrococci.

In 1922, working with hemolytic Staphylococci, Julienelle (39) did not find any correlation between hemolytic activity and biochemical reactions, but in 1937 (40) he re-discovered the correlation between mannitol fermentation and pathogenicity. Most of the nonpathogenic strains did not ferment mannitol, whereas pathogenic strains showed fermentation in intervals ranging from less than 24 hours to 3 and 5 days.

The credit must go to Abd-el-Malek and Gibson (1) for introducing a new approach to the classification of Staphylococci and Micrococci and particularly for showing the value of acetoin production as a distinguishing character. After studying some 799 strains mainly from dairy sources, they concluded that Staphylococci and Micrococci had been indiscriminately named and that both were members of a large group which they called the Staphylococcus-Micrococcus complex. They

did, however, distinguish between a Staphylococcus group, a Micrococcus group, and an intermediate group. The Staphylococcus group fermented sugars and were sensitive to heat. The intermediate group did not form acid from sugars, and the Micrococci were resistant to heat and produced acid from sugars. Nevertheless they suggested that these three groups formed a continuous series.

Shaw, Stit and Cowan (57) were also unable to find sufficient evidence for separating Staphylococcus from Micrococcus. Therefore they combined all the organisms into one group, Staphylococcus. They did this in the belief that the genus Micrococcus was invalid. Also, the heat resistance test used by Abd-el-Malik was not a reliable test to give reproducible results, but they agreed that the ability or inability to ferment sugars was a good starting point in the classification. Their classification has been called by Cowan (18) a 'lumpers' classification. They put all the Gram positive, catalase positive into one genus, Staphylococcus, and further subdivisions were made on one or two criteria. This is indeed an example of bad classification, especially when applied to very similar groups of organisms, such as Staphylococcus and Micrococcus.

In the middle Fifties there was renewed interest in the classification of Staphylococcus, and an interesting collection of papers was published by Van Eseltine (70), Thatcher and Simon (68) supported the non-separation of Staphylococci and Micrococci, and Evans, Bradford and Niven (23) and Breed (11) supported the separation of these two

groups of organisms. Van Eseltine (70) supported Rahn's view that any line of demarcation between these organisms as suggested by Hucker (37) was an arbitrary one, as one could see every combination of characters. Thatcher and Simon (68) also stressed the inadvisability of separating this group of organisms as they did not see any scheme of consistent determinative properties of Micrococci for positive recognition of the group.

Evans et al. (23) suggested that Staphylococci should be separated from Micrococci on the basis of the ability of the former organisms to grow and form acid anaerobically in a medium containing glucose. This classification formed the basis of that used by Breed (11) when he revised the classification of the Micrococci in Bergey's Manual (1957) and reintroduced the genus Staphylococcus.

In 1959 Hill (31) examined the strains studied by Shaw et al. (57) along with some newly isolated strains. He analysed his results by the methods of numerical taxonomy advocated by Sneath (61). He suggested that both coagulase positive and coagulase negative, acetoin positive strains should be regarded as Staphylococci, and coagulase negative and acetoin negative should be called Micrococci. Pohja and Gyllenberg (53) using similar methods of numerical taxonomy, suggested limiting the genus Staphylococcus to the acetoin producing, Gram positive and catalase positive cocci.

In 1965 the Subcommittee on Taxonomy of Staphylococci and Micrococci (67) concluded that the genera Micrococcus and Staphylococcus

belong to the family Micrococcaceae. The genus Staphylococcus contains rather two homogeneous species Staphylococcus aureus and Staphylococcus epidermidis and the genus Micrococcus contain two recognizable species, M. luteus and M. roseus. They also emphasized that other species in these genera have not been adequately characterized.

Classification by analysis of chemical composition.

With the increasing number of strains and species and the intermediate forms discovered every day, the classification of micro-organisms is becoming more complicated and difficult to handle with the conventional methods used so far. The trend at present is to analyze all the components or some of the components of the organisms. For an individual investigator, choice of methods is to some extent conditional because of availability of equipment. So far, column chromatography, paper chromatography and spectrophotometric methods have been used for this kind of study. The new methods which are just being introduced for chemical analysis are gas chromatography and gel electrophoresis. Other than the work presented in this thesis, these methods have contributed little to the classification of Micrococci and Staphylococci so far, although they have been occasionally used in attempts to establish their worth with other microorganisms.

In 1963 Oyama (50) was the first to use gas chromatography on bacteria. However, he did not use it for classification purposes. He used the pyrolysis products of bacteria or substances of biological origin when working on the development of methods to determine the

possible existence of life in extraterrestrial environments. Abel et al. (2) were the first to employ gas liquid chromatography for classification of bacteria. Their work was limited to analysis of methyl esters of fatty acids produced by the bacterial cells. They were able to differentiate between the families of Enterobacteriaceae, Bacillaceae, Micrococcaceae and Parabacteriaceae. The identification of genera and species was difficult with this method. Reiner (55) used gas chromatography to distinguish between strains of bacteria of similar antigenic or pathogenic character. For analysis he used pellets of lyophilized bacteria weighing 200 - 800 μgm . They were pyrolyzed in an inert atmosphere on a nickel filament at 850°C for 10 seconds. The programmed temperature range used for pyrolysis was from 50° to 240°C at a linear rate of increase of 12° per minute. The column consisted of 8 foot-long copper tubing packed with 15% carbowax coated on 80/90 mesh 'Anakron ABS'. Nitrogen gas under a pressure of 16 lb/in^2 was used as a carrier gas. By the gas chromatographic technique, the products of thermal decomposition are measurable at the instant that the vapors are swept into the detector.

Each strain produced its own unique chromatogram of pyrolysis products. Different cultures of the same strain yielded similar profiles. Furthermore, he found that results from two successive analyses on equal portions of the same sample showed remarkable reproducibility. He performed his analysis on E. coli (18 different antigenic strains), Shigella, (1 strain), Streptococcus pyogenes (4 types) and Mycobacteria

(10 different pathogenic and non-pathogenic forms). All these organisms showed similar profiles but differed slightly from each other in their peak ratios.

In 1966 Hanis et al. (30) identified bacteria by analysis of their metabolic products by gas chromatography. The products were obtained by extraction of bacteria with ether. The approach of these workers to the rapid detection or differentiation of microorganisms was based on the fact that the volatile bacterial products or the other products which can be converted into volatile derivatives can be found by the use of highly sensitive detectors. They made use of two types of detectors, i.e. flame ionization detector and electron capture detector. Among the products detected were compounds with the chromatographic characteristics of acetic, propionic and butyric acids, ethyl alcohol, diacetyl, acetoin and 2 - 3 - butanediol. The peak areas were measured and arranged in the order of decreasing areas to yield a signature for each bacterial strain. Different genera and species and strains of the same species differ from each other in their signatures. This clearly proves the usefulness of gas chromatography for identifying or classifying very similar groups of organisms. In order to test the validity of this method in use for classification, Bawdon and Bassette (9) applied it for the differentiation of E. coli and Aerobacter aerogenes. The cultures of each were prepared in heat and vacuum treated milk, and incubated for 30 hours at 35°C. The milk was heated and vacuum treated in order to remove all the volatile components. The samples from these inoculates

were then analyzed by gas chromatography and the chromatograms obtained were differentiated on the basis of peaks produced at different times. There were some peaks which were present in E. coli and absent in A. aerogenes and vice versa.

So far, the identification of bacteria by gas chromatographic analysis of metabolic products required the use of pure cultures. In addition, it was necessary to remove all the volatile materials from the media prior to use. However, Cecchini and O'Brian (14) identified E. coli from other bacteria by gas chromatographic analyses of culture media. The cultures were incubated in a defined medium at 40°C for 8 hours on a shaker. After incubation, the cultures were centrifuged and the supernatant fluids were analysed.

These findings indicate that pyrolysis of substances of considerable complexity can form a new basis of taxonomy in the bacteriological discipline, and that when one is dealing with groups with slight differences or with intermediary forms, gas chromatography might be a useful adjunct to present techniques.

Classification with the aid of computers (numerical taxonomy).

Sneath (61) looked at the classification of bacteria and felt that there was a need for some mechanical aid to sort the data. This was because of the difficulty in organizing the results of all the strains in all the tests which led to the inability to draw any conclusions or to separate the strains into valid taxonomic groups. Sneath further advocated that the ideal classification should have four

main features. First, there should be the greatest amount of data. Second, the overall similarity must be considered. Third, equal weight should be given to all the features. Fourth, division into taxonomic groups should be made upon the correlated features. Prior to Sneath, the first taxonomist who conceived of the use of every feature impartially and with equal weight was Adanson (1763). His views were not accepted since his contemporaries felt that some features were more important than others. Sneath revived Adanson's views. He felt that overall similarity was the key for the development of these principles and it could not be calculated in any other way than by the numerical method. On the other hand, the massive data accumulating in the laboratory could never be adequately analyzed without mechanical aid. The mechanical aid he used was a correctly instructed computer. The basis he used to instruct the computer was as follows: the features between individuals were put into three classes. Class A included the features possessed by the first but not the second individual, Class B included the features possessed by both individuals, and Class C included the features possessed by the second but not by the first individual. The overall similarity was represented by the fraction $B/A+B+C$ and theoretically varied from 1.0 where the individuals were identical in all respects to zero where very different kinds of organisms were compared. In the program used for classification, Sneath compared each pair of strains in all possible combinations. The number of similar features in which both strains of the pair scored positive and the number of different features in which one strain scored positive and

the other negative were counted. The machine did not make any comparison of a negative with a negative nor of a positive with a negative. The S (similarity) value was calculated as shown above. Then the machine sorted the strains into groups, working in small steps of S. Any pair of strains with S values over 0.99 were placed together and these groups (each consisting of almost identical strains) were printed. The above step was repeated for an S. value of over 0.98, 0.97, 0.96 and so on until all the strains had been grouped and printed. Up to this point it was only possible to ascertain the groups that were present and their relationship to each other. The machine was then instructed to find intergroup similarities. The results obtained from the above instruction gave information about two groups, specifying whether these two groups were sufficiently similar to belong to one genus, or so different as to be two different genera. Furthermore, Sneath suggested that workers should avoid using tests which readily separate the groups of organisms.

Choice of the most suitable method of detection of relationships and clusters is still a problem. As suggested by the Subcommittee on Taxonomy of Staphylococci and Micrococci (67), the species in those genera other than S. aureus, S. epidermidis, M. luteus and M. roseus have not been adequately characterized. The work presented in this thesis therefore was undertaken with the idea of investigating the relationships of certain species of Micrococci to currently recognizable species.

II

CLASSIFICATION OF STAPHYLOCOCCI AND MICROCOCCI ON THE BASIS OF MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS

LIST OF DIFFERENT CHARACTERS

Morphological characteristics

Shape of organisms.

Biochemical tests.

Gram stain (cytochemical)

Deoxyribonuclease

Coagulase

Penicillinase

Phosphatase

Salt tolerance (5%)

Salt tolerance (7%)

Salt tolerance (10%)

Azide tolerance (.02%)

Azide enhancement

KCN tolerance (.5%)

KCN enhancement

Mannitol fermentation

Catalase

Oxidation

Fermentation

NH₄ production (anaerobic)

NH₄ production (aerobic)

Biochemical tests (Contd.)

Nitrate reduction

Casein hydrolysis

Hemolysis

Lipolysis

Gelatin liquefaction

Starch hydrolysis

Hippurate production

Acetoin production

Methyl red

Oxidase

METHODS AND MATERIALS

Sixty-two strains of Gram positive cocci (27 Staphylococci and 35 Micrococci) and 52 strains of Gram negative rods were in the study. The Staphylococci were isolates from ear, nose, throat and ulcer swabs from patients at the University of Alberta Hospital. Micrococci NCIB which are available from the National Collection of Industrial Bacteria in Aberdeen, Scotland, were obtained through the courtesy of the Department of Microbiology, University of Alberta, and Micrococci CCM were obtained from the Czechoslovak Collection of Microorganisms at J. E. Purkyne University in Brno, Czechoslovakia. Gram negative organisms which were mainly used for comparison purposes were isolates of urine specimens from patients at the University of Alberta Hospital.

During the course of the study, the organisms were maintained on nutrient agar slants at 4°C. The slants were prepared in ½ oz. round, screw-capped bottles and transfers were made at 2 to 3 month intervals. The strains of Micrococci and Staphylococci were also lyophilized in milk for long-term storage.

Morphological Observations

The medium which was used throughout for the growth of the organisms was nutrient agar + 5% yeast extract. Except where otherwise stated, this will be referred to as Nutrient Yeast Agar: NYA. It was prepared by adding 1.5% Difco agar to nutrient broth (Oxoid #2).

Staphylococci and Gram negative organisms were examined after 48 hours of incubation at 37°C. Micrococci were examined after 48 hours of incubation at 37°C.

Biochemical Tests.

All the media for the growth of the organisms for physiological and biochemical tests were supplemented with .5% yeast extract.

Gram stain.

The organisms were stained by Kopeloff's (44) modified method of Gram stain and were examined for Gram stain reaction.

Coagulase test.

The coagulase test was performed by a tube method. Rabbit plasma was diluted in nutrient broth to give a final dilution of 1/10. To 0.5ml of this diluted plasma, 0.1 ml of a 24-hour broth culture of the organisms was added. The tubes were examined for coagulum after 1, 2, 3, 6, 8, and 12 hours of incubation at 37°C. Most of the tubes in which the test was positive showed a coagulum in 4 hours. A few positives were detected after 8 hours of incubation.

Catalase test.

(a) Slide test: a small amount of the culture grown on nutrient agar slants for 24 hours was put on a slide. Hydrogen peroxide 3% (0.1 ml) was dropped on the culture and examined for the evolution of bubbles of gas.

(b) Tube test: this test was performed by immersing a small amount of the culture picked with a platinum wire in 2 ml of 3% H₂O₂

in a tube. Bubbles of gas which were formed could be seen easily as they moved to the top through the H_2O_2 solution.

Deoxyribonuclease production.

Agar plates were made with BBL DNase medium at a concentration of 40 gm of medium per litre of distilled water. The organisms were streaked in a line across the surface of the plates and incubated until good growth had been achieved. The plates were flooded with normal hydrochloric acid and examined for clearing around the colonies. The clearing was caused by the breakdown of DNA to polyribonucleotides which do not precipitate with acid.

Penicillinase production.

Organisms were inoculated on sterile Millipore filters - pore size 0.45μ - 55 mm diameter, placed on the surface of nutrient agar plates containing 2 units of penicillin per ml. The filter papers were removed after 24 hours of incubation at $37^{\circ}C$ and the plates were flooded with an overnight culture of the indicator Staphylococcus aureus (diluted 1/10). Excess fluid was removed and the plates were re-incubated for a further 24-hour period and examined for penicillinase production. Growth of the organism, which was penicillin sensitive, indicated the production of penicillinase (33).

Phosphatase production.

Ability to produce phosphatase was tested on a medium containing (% W/V); peptone, 0.5; Lab-Lemco, 0.5; sodium chloride, 0.5; agar 1.5.

To 100 mls of the above molten basal medium, 4 tablets of buffered phenothalein diphosphate were added and the pH of the medium was adjusted to 9.4 (42). The plates were inoculated in duplicate. One set of plates was checked for phosphatase activity after 18 - 24 hours of incubation at 37°C. The other set of plates was checked after 4 days of incubation at 37°C. The release of free phenolthalein was detected by exposing the incubated plates to ammonia fumes. Phosphatase-producing colonies turned deep pink on exposure (7).

Lipase production.

The organisms were streaked on NYA plates containing 1% fat (olive oil). After 24 hours a saturated solution of CuSO_4 was added to the plates and the excess was decanted. Lipolytic activity was examined after rinsing the plates gently with water. When the fat has been attacked, bluish green streaks of insoluble copper soaps appeared around the streaks (10).

Casein hydrolysis.

Casein agar plates (6) were inoculated and were examined after 10 days of incubation at 37°C. At the end of this period the plates were flooded with acid mercuric chloride (25) to distinguish between true proteolysis and clearing of milk due to solubilization of milk protein by alkaline end products of metabolism. Decrease in clearing area on flooding the plate with mercuric chloride showed that casein had not been hydrolyzed.

Gelatin liquefaction.

Gelatin liquefaction was detected by the method of Frazier (25). The organisms were grown on gelatin agar medium and after 4 days of incubation at 37°C the plates were flooded with an acid solution of bichloride of mercury (HgCl_2) 15 gm, HCl (conc.) 20 cc and water 100 cc. Gelatin liquefaction was indicated by a clear zone around the colonies.

Ammonia production.

L-arginine was used as a nitrogen source for the growth of the organisms. Ammonia was detected by adding 0.5 ml of Nessler's reagent to 1 ml of the culture supernatant obtained after incubating the organisms in L-arginine broth aerobically and anaerobically for 14 days at 30°C. Rust brown precipitate marked the presence of ammonia.

Voges-Proskauer reaction.

The organisms were grown in phosphate-free 2% glucose broth for 14 days at 30°C. The presence of acetylmethyl-carbinol (acetoin) was detected by the method of Barritt (8).

Methyl red test.

To a part of the suspension of organisms grown in MR and V-P medium for 3 days, 5 drops of 0.04% of methyl red solution (0.04 gm methyl red + 40 ml of absolute ethanol + 60 ml of distilled water) was added (20).

Hemolysis.

The organisms were tested for hemolysis on 5% rabbit blood agar plates.

Oxidation and fermentation tests.

(a) Tube Test: the tubes containing Hugh and Leifson's media + bromothymol blue as an indicator were inoculated vertically through the agar by means of a straight wire.

(b) Plate Test: the organisms were streaked on plates containing the same nutrient broth as for the tube test, solidified with 1.5% agar.

For oxidation both the plates and tubes were examined after a week of incubation at 37°C. For fermentation they were examined after 2 weeks of incubation at 37°C in anaerobic jars. In either case, the positive reaction was marked by a change of color of medium from original blue to green or yellow, caused by acid production.

Oxidase test.

(a) Plate Test: a loopful of overnight culture of organisms was placed on a filter paper in a petri dish. Two drops of tetramethyl paraphenylene diamine were dropped on each of the cultures and examined for color change (45).

(b) Tube Test: the organisms were grown on NYA medium for 20 hours at 37°C, and the plates were washed with phosphate buffer (pH 7.0, 0.025 M). All the suspensions were diluted in order to give an optical density of .7. To 1.3 ml of the above diluted suspension, .23 ml of tetramethyl paraphenylene diamine was added and examined for color change in the first 2 minutes, and 30 minutes at 25°C. The positive test was marked by the appearance of pink color. The results of the plate test were not sufficiently reproducible, and those from the tube test were entered in the Tables.

Starch hydrolysis.

The organisms were grown on starch agar plates for 7 days. The plates were flooded with Lugol's iodine (iodine 5 gm + potassium iodide 10 gm + 100 ml distilled water) and examined for starch hydrolysis. Non-hydrolyzed areas turned blue, while hydrolysis was indicated by clearing around the colonies. In some cases restricted zones of hydrolysis were produced so that it was necessary to scrape off the colonies to see the clear zones underneath.

Hippurate hydrolysis.

The organisms were grown in hippurate broth medium for 14 days at 30°C. Hippurate hydrolysis was detected by adding 1.5 ml of 50% v/v concentrated H_2SO_4 to 1 ml of the culture supernatant and allowing it to stand for 4 hours. Free benzoic acid which precipitated as fine crystals, indicated hippurate hydrolysis.

Nitrate reduction.

Nitrate reduction was detected by adding 1 ml of nitrite reagent A (0.8% sulphanilic acid in 5 N - acetic acid) followed by 1 ml of reagent B (0.5% α -naphthylamine in 5N - acetic acid) to a suspension of organisms obtained after 14 days of incubation in nitrate broth at 30°C (20). The appearance of red color indicated reduction to nitrite and absence of color change was indicative of either the absence of nitrite or that nitrite had been further reduced to gaseous nitrogen or hydroxylamine. The appearance of a pink color on addition of zinc dust to the cultures negative for nitrite indicated presence of unchanged nitrate.

Salt tolerance.

A suspension of organisms in distilled water was prepared and adjusted so that 1 drop (0.02 ml from a calibrated dropping pipette) gave 15 - 20 colonies on a plate without added sodium chloride. Salt tolerance was measured by the ability of organisms to grow on plates supplemented with 5%, 7% and 10% sodium chloride. The results were recorded after 48 hours of incubation at 37°C.

Growth in the presence of azide and cyanide.

The organisms were grown on two sets of NYA plates, one set containing 0.02% sodium azide and the other 0.5% potassium cyanide. The size of the inoculum and optical density of the suspensions were fixed so as to give 15 - 20 colony-forming units per drop of inoculum. The plates were examined for growth and for stimulation of growth by comparing with control plates without azide or cyanide.

RESULTS

Morphological characteristics.

Microscopic observations of the stained smears of colonies revealed that all the organisms which were spherical in shape were Gram positive and all the rods were Gram negative.

Biochemical characteristics.

Tables I, II, III summarize the results of physiological and biochemical studies. In order to draw any valid conclusions from these results, the data were analyzed with the aid of a computer.

Analysis of data with the aid of a computer.

The principles of microbial classification and analysis of results with the aid of a computer (numerical taxonomy) were set forth by Sneath in 1957 (61), although the underlying concepts dated back to Michel Adanson in 1765. Sneath (62) emphasized that the ideal classification is that based on overall similarity in which every feature of the organism carries an equal weight. The program for natural clusters written by J. Alan George, J. W. Carmichael and R. S. Julius (28) has been used for the analysis of data. As in Sneath's procedure, every feature of the organism has been given an equal weight.

Data from biochemical and cytochrome patterns (Part II) were tabulated. The same data were punched on cards and the computer was programmed according to the problem being studied. Like most numerical procedures, the first step in the compression of data is the determination of some single value for each pair of OTU's (Operational Taxonomic Units)

[illegible]

which reflects their overall relationship. This overall relationship is represented by a coefficient of similarity, the value of which is a function of the distance between pairs of OTU's with N attributes or characters. There are various measures of similarity as outlined by Sokal (63). In this program the relative distance between two values (V) is given by:

$$d = \left| V_i - V_j \right| / (V_{\max} - V_{\min})$$

Where V max and V min are the largest and smallest values of the measure over the set of OTU's, then the similarity or proximity is determined as follows:

$$s = 1 - d$$

For the similarities between OTU's as calculated above, the attribute values are the coordinates determining points in space. When there are a large number of OTU's with a considerable number of attributes, it is hard to visualize the configuration of these points in the attribute space. This led to the need for finding natural clusters of points. Therefore the next part of the program constructs a histogram of ranked similarities on the basis of which the natural clusters are formed. The strategies used in the formation of clusters are as follows:

Strategy 1. If there are any natural clusters in the configuration, the closest pair of points will be members of one of them. In this way additional clusters are also found.

Strategy 2. An additional member is added to the cluster on the basis of its closeness to any point in the cluster.

Strategy 3. A point which is excluded from a cluster but is closer to

the cluster than to any other point is a satellite of the cluster.

Since there may be clusters of different sizes, the whole population of organisms is examined at different levels of resolution. The highest level of resolution has a similarity value, i.e. 10% of the way down from the top of the rank ordered list and the lowest level of resolution has a similarity value which is 5% of the way up from the bottom of the list. The data were analyzed at twelve different levels of resolution.

Exclusion strategies.

There are four exclusion strategies. A point is not included in a cluster if:

1. Its average distance from the points in the cluster indicates a discontinuity in closeness. This is called the Average Linkage criterion.
2. Its distance from the closest point in the cluster indicates a discontinuity in closeness. This is called the Best Link criterion.
3. The cluster is non-spherical and has grown large in some other direction before the point is considered for admission.
4. Points which are not close to any other points are one-member clusters.

Results.

The above procedure was used for the investigation of the clustering of 62 strains of Staphylococci and Micrococci on the basis of thirty-one characters. The results so obtained are shown in Tables IV - XI.

TABLE IV

RESULTS FROM COMPUTER ANALYSIS OF BIOCHEMICAL
TESTS AND CYTOCHROME PATTERNS AT:RESOLUTION LEVEL 1 Average link parameter 0.930
Single link parameter 0.930

Cluster No.	Organisms
1	<u>M. luteus</u> CCM 855 <u>M. luteus</u> CCM 266 <u>M. luteus</u> CCM 1335 <u>M. luteus</u> CCM 852
2	<u>M. luteus</u> CCM 248 <u>M. luteus</u> CCM 331 <u>M. luteus</u> CCM 622
3	<u>M. luteus</u> CCM 309 <u>M. luteus</u> CCM 210 <u>M. luteus</u> CCM 852
4	<u>S. aureus</u> 42D <u>S. aureus</u> 29 <u>S. aureus</u> 52 <u>S. aureus</u> 2051
5	<u>S. aureus</u> 2022 <u>S. aureus</u> 2053 <u>S. aureus</u> 2031 <u>S. aureus</u> 2030 <u>S. aureus</u> 2052 <u>S. aureus</u> 187 <u>S. aureus</u> 2057
6	<u>S. albus</u> 12897 <u>S. albus</u> 12768 <u>S. albus</u> 901 <u>S. albus</u> 12761

(contd.)

TABLE IV (Contd.)

Cluster No.	Organisms
7	<u>M. violagabriellae</u> Parent <u>M. violagabriellae</u> Sp <u>M. violagabriellae</u> 2a <u>S. albus</u> 12731
8	<u>S. aureus</u> 2024 <u>S. aureus</u> 2034 <u>S. aureus</u> 2057 <u>S. aureus</u> 2061 <u>S. aureus</u> 2051 <u>S. aureus</u> 8511
9	<u>S. albus</u> 12761 <u>S. albus</u> 12725 <u>S. albus</u> 12768
Satellite of Cluster 8	<u>S. aureus</u> 8511
Satellite of Cluster 8	<u>S. aureus</u> 3a
Satellite of Cluster 8	<u>M. violagabriellae</u> L.P.
Satellite of Cluster 8	<u>S. aureus</u> 6
Satellite of Cluster 7	<u>S. albus</u> 12731
Satellite of Cluster 1	<u>M. luteus</u> CCM 852
Satellite of Cluster 1	<u>M. roseus</u> NCIB 146
Satellite of Cluster 1	<u>M. luteus</u> CCM 622

(Contd.)

TABLE IV (Contd.)

Cluster No.	Organisms
10	<u>M. varians</u> CCM 1046 <u>M. flavus</u> NCIB 8166 <u>M. luteus</u> CCM 852
Satellite of Cluster 1	<u>M. lysodeikticus</u> NCIB 9278
Satellite of Cluster 2	<u>M. luteus</u> CCM 265
Satellite of Cluster 1	<u>M. luteus</u> CCM 853
Organisms not placed in Cluster	<u>S. aureus</u> 2046 <u>M. tetragenus</u> <u>M. halophilus</u> <u>M. cyaneus</u> NCIB 9148 <u>M. cyaneus</u> CCM 856 <u>M. freudenreichii</u> NCIB 2699 <u>M. aurantiacus</u> NCIB 8609 <u>M. candidus</u> NCIB 8610 <u>M. roseus</u> NCIB 8175 <u>M. roseus</u> CCM 706 <u>M. luteus</u> CCM 250 <u>M. varians</u> CCM 884 <u>M. sodonensis</u> NCIB 8854 <u>M. luteus</u> NCIB 8165 <u>M. luteus</u> CCM 559 <u>M. luteus</u> CCM 247 <u>M. luteus</u> CCM 851 <u>M. conglomeratus</u> NCIB 2677 <u>M. cereus</u> <u>M. saprophiticus</u>

TABLE V

RESULTS FROM COMPUTER ANALYSIS OF BIOCHEMICAL
TESTS AND CYTOCHROME PATTERNS AT:

RESOLUTION LEVEL 2 Average link parameter 0.880
 Single link parameter 0.905

Cluster No.	Organisms
1	<u>M. luteus</u> CCM 855 <u>M. luteus</u> CCM 266 <u>M. luteus</u> CCM 1335 <u>M. luteus</u> CCM 852 <u>M. roseus</u> NCIB 146 <u>M. luteus</u> CCM 622 <u>M. luteus</u> CCM 248
2	<u>M. luteus</u> CCM 248 <u>M. luteus</u> CCM 331 <u>M. luteus</u> CCM 622
3	<u>M. luteus</u> CCM 309 <u>M. luteus</u> CCM 210 <u>M. luteus</u> CCM 852
4	<u>S. aureus</u> 42 D <u>S. aureus</u> 29 <u>S. aureus</u> 52 <u>S. aureus</u> 2051 <u>S. aureus</u> 2034 <u>S. aureus</u> 2024 <u>S. aureus</u> 2057 <u>S. aureus</u> 2061 <u>S. aureus</u> 8511 <u>S. aureus</u> 2053 <u>S. aureus</u> 2022 <u>S. aureus</u> 2031 <u>S. aureus</u> 2030 <u>S. aureus</u> 2052 <u>S. aureus</u> 187 <u>S. aureus</u> 3 A

(Contd.)

TABLE V (Contd.)

Cluster No.	Organisms
5	<u>S. albus</u> 12897 <u>S. albus</u> 12768 <u>S. albus</u> 901 <u>S. albus</u> 12761 <u>S. albus</u> 12725
6	<u>M. violagabriellae</u> Parent <u>M. violagabriellae</u> SP <u>M. violagabriellae</u> 2a <u>S. albus</u> 12731 <u>S. albus</u> 12768
Satellite of Cluster 4	<u>S. aureus</u> 3a
Satellite of Cluster 4	<u>M. violagabriellae</u> L.P.
Satellite of Cluster 4	<u>S. aureus</u> 6
7	<u>M. varians</u> CCM 1046 <u>M. flavus</u> NCIB 8166 <u>M. luteus</u> CCM 852
Satellite of Cluster 1	<u>M. lysodeikticus</u> NCIB 9278
Satellite of Cluster 2	<u>M. luteus</u> CCM 265
Satellite of Cluster 1	<u>M. luteus</u> CCM 853
Organisms not placed in Clusters	<u>S. aureus</u> 2046 <u>M. tetragenus</u> <u>M. halophilus</u> <u>M. cyaneus</u> NCIB 9148 <u>M. cyaneus</u> CCM 856

(Contd.)

TABLE V (Contd.)

Cluster No.	Organisms
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Organisms not placed in clusters (contd.)

<u>M. freudenreichii</u>	NCIB 2699
<u>M. aurantiacus</u>	NCIB 8609
<u>M. candidus</u>	NCIB 8610
<u>M. roseus</u>	NCIB 8175
<u>M. roseus</u>	CCM 706
<u>M. luteus</u>	CCM 250
<u>M. varians</u>	CCM 884
<u>M. sodonensis</u>	NCIB 8854
<u>M. luteus</u>	NCIB 8165
<u>M. luteus</u>	CCM 559
<u>M. luteus</u>	CCM 247
<u>M. luteus</u>	CCM 851
<u>M. conglomeratus</u>	NCIB 2677
<u>M. cereus</u>	
<u>M. saprophiticus</u>	

TABLE VI

RESULTS FROM COMPUTER ANALYSIS OF BIOCHEMICAL
TESTS AND CYTOCHROME PATTERNS AT:

RESOLUTION LEVEL 3 Average link parameter 0.830
 Single link parameter 0.880

RESOLUTION LEVEL 4* Average link parameter 0.780
 Single link parameter 0.855

Cluster No.	Organisms
1	<u>M. luteus</u> CCM 855 <u>M. luteus</u> CCM 266 <u>M. luteus</u> CCM 1335 <u>M. luteus</u> CCM 852 <u>M. roseus</u> NCIB 146 <u>M. luteus</u> CCM 622 <u>M. luteus</u> CCM 248 <u>M. luteus</u> CCM 331 <u>M. luteus</u> CCM 309 <u>M. luteus</u> CCM 210 <u>M. lysodeikticus</u> NCIB 9278 <u>M. luteus</u> CCM 265 <u>M. luteus</u> CCM 853 <u>M. roseus</u> CCM 706
2	<u>S. aureus</u> 42 D <u>S. aureus</u> 29 <u>S. aureus</u> 52 <u>S. aureus</u> 2051 <u>S. aureus</u> 2034 <u>S. aureus</u> 2024 <u>S. aureus</u> 2057 <u>S. aureus</u> 2061 <u>S. aureus</u> 8511 <u>S. aureus</u> 2053 <u>S. aureus</u> 2022 <u>S. aureus</u> 2031 <u>S. aureus</u> 2030 <u>S. aureus</u> 2052 <u>S. aureus</u> 187 <u>S. aureus</u> 3A <u>M. violagabriellae</u> L.P. <u>S. aureus</u> 6 <u>S. albus</u> 12725

(Contd.)

TABLE VI (Contd.)

Cluster No.	Organisms	
3	<u>S. albus</u>	12897
	<u>S. albus</u>	12768
	<u>S. albus</u>	901
	<u>S. albus</u>	12761
	<u>S. albus</u>	12725
	S.P.	
4	<u>M. violagabriellae</u>	Parent
	<u>M. violagabriellae</u>	SP
	<u>M. violagabriellae</u>	2a
	<u>S. albus</u>	12731
	<u>S. albus</u>	12768
5	<u>M. varians</u>	CCM 1046
	<u>M. flavus</u>	NCIB 8166
	<u>M. luteus</u>	CCM 852
Organisms not placed in cluster		
	<u>S. aureus</u>	2046
	<u>M. tetragenus</u>	
	<u>M. halophilus</u>	
	<u>M. cyaneus</u>	NCIB 9148
	<u>M. cyaneus</u>	CCM 856
	<u>M. freudenreichii</u>	NCIB 2699
	<u>M. aurantiacus</u>	NCIB 8609
	<u>M. candidus</u>	NCIB 8610
	<u>M. roseus</u>	NCIB 8175
	<u>M. roseus</u>	CCM 706
	<u>M. luteus</u>	CCM 250
	<u>M. varians</u>	CCM 884
	<u>M. sodonensis</u>	NCIB 8834
	<u>M. luteus</u>	NCIB 8165
	<u>M. luteus</u>	CCM 559
	<u>M. luteus</u>	CCM 247
	<u>M. luteus</u>	CCM 851
	<u>M. conglomeratus</u>	NCIB 2677
	<u>M. cereus</u>	
	<u>M. saprophiticus</u>	

* Tables corresponding to resolution levels 3 and 4 are exactly similar.

TABLE VII

RESULTS FROM COMPUTER ANALYSIS OF BIOCHEMICAL
TESTS AND CYTOCHROME PATTERNS AT:

RESOLUTION LEVEL 5 Average link parameter 0.730
 Single link parameter 0.830

RESOLUTION LEVEL 6* Average link parameter 0.680
 Single link parameter 0.805

Cluster No.	Organisms
1	<u>M. luteus</u> CCM 855 <u>M. luteus</u> CCM 266 <u>M. luteus</u> CCM 1335 <u>M. luteus</u> CCM 852 <u>M. roseus</u> NCIB 146 <u>M. luteus</u> CCM 622 <u>M. luteus</u> CCM 248 <u>M. luteus</u> CCM 331 <u>M. luteus</u> CCM 309 <u>M. luteus</u> CCM 210 <u>M. lysodeikticus</u> NCIB 9278 <u>M. luteus</u> CCM 265 <u>M. luteus</u> CCM 853 <u>M. roseus</u> CCM 706 <u>M. luteus</u> CCM 559 <u>M. sodonensis</u> NCIB 8854 <u>M. luteus</u> CCM 247 <u>M. varians</u> CCM 1046 <u>M. flavus</u> NCIB 8166 <u>M. saprophiticus</u>
2	<u>S. aureus</u> 42D <u>S. aureus</u> 29 <u>S. aureus</u> 52 <u>S. aureus</u> 2051 <u>S. aureus</u> 2034 <u>S. aureus</u> 2024 <u>S. aureus</u> 2057 <u>S. aureus</u> 2061 <u>S. aureus</u> 8511 <u>S. aureus</u> 2053 <u>S. aureus</u> 2022 <u>S. aureus</u> 2031 <u>S. aureus</u> 2030 <u>S. aureus</u> 2052

(Contd.)

TABLE VII (Contd.)

Cluster No.	Organisms
	<u>S. aureus</u> 187 <u>S. aureus</u> 3a <u>M. violagabriellae</u> LP <u>S. aureus</u> 6 <u>S. albus</u> 12725
3	<u>S. albus</u> 12897 <u>S. albus</u> 12768 <u>S. albus</u> 901 <u>S. albus</u> 12761 <u>S. albus</u> 12725 <u>M. violagabriellae</u> SP <u>M. violagabriellae</u> Parent <u>M. violagabriellae</u> 2a <u>S. albus</u> 12731 <u>M. tetragenus</u> <u>S. aureus</u> 2046 <u>M. halophilus</u>
Organisms not placed in clusters	<u>M. halophilus</u> <u>M. cyaneus</u> NCIB 9148 <u>M. cyaneus</u> CCM 856 <u>M. freudenreichii</u> NCIB 2699 <u>M. aurantiacus</u> NCIB 8609 <u>M. candidus</u> NCIB 8610 <u>M. roseus</u> NCIB 8175 <u>M. luteus</u> CCM 250 <u>M. varians</u> CCM 884 <u>M. luteus</u> NCIB 8165 <u>M. luteus</u> CCM 851 <u>M. conglomeratus</u> NCIB 2677 <u>M. cereus</u> <u>M. saprophiticus</u>

* Tables corresponding to resolution levels 5 and 6 are exactly similar.

TABLE VIII

RESULTS FROM COMPUTER ANALYSIS OF BIOCHEMICAL
TESTS AND CYTOCHROME PATTERNS AT:RESOLUTION LEVEL 7 Average link parameter 0.630
Single link parameter 0.780

Cluster No.	Organisms		
1	<u>M. luteus</u>	CCM	855
	<u>M. luteus</u>	CCM	266
	<u>M. luteus</u>	CCM	1335
	<u>M. luteus</u>	CCM	852
	<u>M. roseus</u>	NCIB	146
	<u>M. luteus</u>	CCM	622
	<u>M. luteus</u>	CCM	248
	<u>M. luteus</u>	CCM	331
	<u>M. luteus</u>	CCM	309
	<u>M. luteus</u>		210
	<u>M. lysodeikticus</u>	NCIB	9278
	<u>M. luteus</u>	CCM	265
	<u>M. luteus</u>	CCM	853
	<u>M. roseus</u>	CCM	706
	<u>M. luteus</u>	CCM	559
	<u>M. sodonensis</u>	NCIB	8854
	<u>M. luteus</u>	CCM	247
	<u>M. varians</u>	CCM	1046
	<u>M. flavus</u>	NCIB	8166
	<u>M. saprophiticus</u>		
	<u>M. luteus</u>	CCM	250
2	<u>S. aureus</u>		42D
	<u>S. aureus</u>		29
	<u>S. aureus</u>		52
	<u>S. aureus</u>		2051
	<u>S. aureus</u>		2034
	<u>S. aureus</u>		2024
	<u>S. aureus</u>		2057
	<u>S. aureus</u>		2061
	<u>S. aureus</u>		8511
	<u>S. aureus</u>		2053
	<u>S. aureus</u>		2022
	<u>S. aureus</u>		187
	<u>S. aureus</u>		3A
	<u>M. violagabriellae</u>	LP	
	<u>S. aureus</u>		6
	<u>S. albus</u>		12725

TABLE VIII (Contd.)

Cluster No.	Organisms
3	<u>S. albus</u> 12897 <u>S. albus</u> 12768 <u>S. albus</u> 901 <u>S. albus</u> 12761 <u>S. albus</u> 12725 <u>M. violagabriellae</u> SP <u>M. violagabriellae</u> Parent <u>M. violagabriellae</u> 2a <u>S. albus</u> 12731 <u>M. tetragenus</u> <u>S. aureus</u> 2046 <u>M. halophilus</u>
Link to cluster 2	<u>S. aureus</u> 2053
Organisms not placed in clusters	<u>M. cyaneus</u> NCIB 9198 <u>M. cyaneus</u> CCM 856 <u>M. freudenreichii</u> NCIB 2699 <u>M. aurantiacus</u> NCIB 8609 <u>M. candidus</u> NCIB 8610 <u>M. roseus</u> NCIB 8175 <u>M. luteus</u> CCM 250 <u>M. varians</u> CCM 884 <u>M. luteus</u> NCIB 8165 <u>M. luteus</u> CCM 851 <u>M. conglomeratus</u> NCIB 2677 <u>M. cereus</u>

TABLE IX

RESULTS FROM COMPUTER ANALYSIS OF BIOCHEMICAL
TESTS AND CYTOCHROME PATTERNS AT:

RESOLUTION LEVEL 8 Average link parameter 0.580
 Single link parameter 0.755

RESOLUTION LEVEL 9* Average link parameter 0.530
 Single link parameter 0.730

Cluster No.	Organisms
1	<u>M. luteus</u> CCM 855 <u>M. luteus</u> CCM 266 <u>M. luteus</u> CCM 1335 <u>M. luteus</u> CCM 852 <u>M. roseus</u> NCIB 146 <u>M. luteus</u> CCM 622 <u>M. luteus</u> CCM 248 <u>M. luteus</u> CCM 331 <u>M. luteus</u> CCM 309 <u>M. luteus</u> CCM 210 <u>M. lysodeikticus</u> NCIB 9278 <u>M. luteus</u> CCM 265 <u>M. luteus</u> CCM 853 <u>M. roseus</u> CCM 706 <u>M. luteus</u> CCM 559 <u>M. sodonensis</u> NCIB 8854 <u>M. luteus</u> CCM 247 <u>M. varians</u> CCM 1046 <u>M. flavus</u> NCIB 8166 <u>M. saprophiticus</u> <u>M. luteus</u> CCM 250
2	<u>S. aureus</u> 42D <u>S. aureus</u> 29 <u>S. aureus</u> 52 <u>S. aureus</u> 2051 <u>S. aureus</u> 2034 <u>S. aureus</u> 2024 <u>S. aureus</u> 2057 <u>S. aureus</u> 2061 <u>S. aureus</u> 8511 <u>S. aureus</u> 2053 <u>S. aureus</u> 2022

(Contd.)

TABLE IX (Contd.)

Cluster No.	Organisms
2	<u>S. aureus</u> 2031 <u>S. aureus</u> 2030 <u>S. aureus</u> 2052 <u>S. aureus</u> 187 <u>S. aureus</u> 3A <u>M. violagabriellae</u> LP <u>S. aureus</u> 6 <u>S. albus</u> 12725 <u>S. albus</u> 12761 <u>S. albus</u> 12768 <u>S. albus</u> 901 <u>M. violagabriellae</u> SP <u>M. violagabriellae</u> Parent <u>M. violagabriellae</u> 2a <u>S. albus</u> 12731 <u>M. tetragenus</u> <u>S. aureus</u> 2046 <u>M. halophilus</u> <u>M. candidus</u> NCIB 8610 <u>M. freudenreichii</u> NCIB 2699
Organisms not placed in clusters	<u>M. cyaneus</u> NCIB 9148 <u>M. cyaneus</u> CCM 856 <u>M. freudenreichii</u> NCIB 2699 <u>M. aurantiacus</u> NCIB 8609 <u>M. roseus</u> NCIB 8175 <u>M. luteus</u> CCM 250 <u>M. varians</u> CCM 884 <u>M. luteus</u> NCIB 8165 <u>M. luteus</u> CCM 851 <u>M. conglomeratus</u> NCIB 2677 <u>M. cereus</u>

* Table corresponding to resolution levels 8 and 9 are exactly similar.

TABLE X

RESULTS FROM COMPUTER ANALYSIS OF BIOCHEMICAL
TESTS AND CYTOCHROME PATTERNS AT:

RESOLUTION LEVEL 10 Average link parameter 0.480
 Single link parameter 0.705

RESOLUTION LEVEL 11* Average link parameter 0.430
 Single link parameter 0.680

Cluster No.	Organisms	
1	<u>M. luteus</u>	CCM 855
	<u>M. luteus</u>	CCM 266
	<u>M. luteus</u>	CCM 1335
	<u>M. luteus</u>	CCM 852
	<u>M. roseus</u>	NCIB 146
	<u>M. luteus</u>	CCM 622
	<u>M. luteus</u>	CCM 248
	<u>M. luteus</u>	CCM 331
	<u>M. luteus</u>	CCM 309
	<u>M. luteus</u>	CCM 210
	<u>M. lysodeikticus</u>	NCIB 9278
	<u>M. luteus</u>	CCM 265
	<u>M. luteus</u>	CCM 853
	<u>M. roseus</u>	CCM 706
	<u>M. luteus</u>	CCM 559
	<u>M. sodonensis</u>	NCIB 8854
	<u>M. luteus</u>	CCM 247
	<u>M. varians</u>	CCM 1046
	<u>M. flavus</u>	NCIB 8166
	<u>M. saprophiticus</u>	
	<u>M. luteus</u>	CCM 250
	<u>M. varians</u>	CCM 884
	<u>M. conglomeratus</u>	NCIB 2677
	<u>M. luteus</u>	CCM 851
2	<u>S. aureus</u>	42D
	<u>S. aureus</u>	29
	<u>S. aureus</u>	52
	<u>S. aureus</u>	2051
	<u>S. aureus</u>	2034
	<u>S. aureus</u>	2024
	<u>S. aureus</u>	2031
	<u>S. aureus</u>	2030
	<u>S. aureus</u>	2052
	<u>S. aureus</u>	

(Contd.)

TABLE X (Contd.)

Cluster No.	Organisms
2	<u>S. aureus</u> 187 <u>S. aureus</u> 3A <u>M. violagabriellae</u> LP <u>S. aureus</u> 6 <u>S. albus</u> 12725 <u>S. albus</u> 12761 <u>S. albus</u> 12768 <u>S. albus</u> 901 <u>M. violagabriellae</u> SP <u>M. violagabriellae</u> Parent <u>M. violagabriellae</u> 2A <u>S. albus</u> 12731 <u>M. tetragenus</u> <u>S. aureus</u> 2046 <u>M. halophilus</u> <u>M. candidus</u> NCIB 8610 <u>M. freudenreichii</u> NCIB 2699 <u>M. aurantiacus</u> NCIB 8609
Organisms not placed in clusters	<u>M. cyaneus</u> NCIB 9148 <u>M. cyaneus</u> CCM 856 <u>M. aurantiacus</u> NCIB 8609 <u>M. roseus</u> NCIB 8175 <u>M. luteus</u> NCIB 8165 <u>M. luteus</u> CCM 851 <u>M. cereus</u>

* Tables corresponding to resolution levels 10 and 11 are exactly similar.

TABLE XI

RESULTS FROM COMPUTER ANALYSIS OF BIOCHEMICAL
TESTS AND CYTOCHROME PATTERNS AT:RESOLUTION LEVEL 12 Average link parameter 0.380
Single link parameter 0.655

Cluster No.	Organisms		
1	<u>M. luteus</u>	CCM	855
	<u>M. luteus</u>	CCM	266
	<u>M. luteus</u>	CCM	1335
	<u>M. luteus</u>	CCM	852
	<u>M. roseus</u>	NCIB	146
	<u>M. luteus</u>	CCM	622
	<u>M. luteus</u>	CCM	248
	<u>M. luteus</u>	CCM	331
	<u>M. luteus</u>	CCM	309
	<u>M. luteus</u>	CCM	210
	<u>M. lysodeikticus</u>	NCIB	9278
	<u>M. luteus</u>	CCM	265
	<u>M. luteus</u>	CCM	853
	<u>M. roseus</u>	CCM	706
	<u>M. luteus</u>	CCM	559
	<u>M. sodonensis</u>	NCIB	8854
	<u>M. luteus</u>	CCM	247
	<u>M. varians</u>	CCM	1046
	<u>M. flavus</u>	NCIB	8166
	<u>M. saprophiticus</u>		
	<u>M. luteus</u>	CCM	250
	<u>M. varians</u>	CCM	884
	<u>M. conglomeratus</u>	NCIB	2677
	<u>M. luteus</u>	CCM	851
	<u>M. luteus</u>	NCIB	8165
	<u>M. roseus</u>	NCIB	8175
	<u>M. cereus</u>		
	<u>M. luteus</u>	CCM	856
2	<u>S. aureus</u>		42D
	<u>S. aureus</u>		29
	<u>S. aureus</u>		52
	<u>S. aureus</u>		2051
	<u>S. aureus</u>		2034
	<u>S. aureus</u>		2024
	<u>S. aureus</u>		2057
	<u>S. aureus</u>		2061

(Contd.)

TABLE XI (Contd.)

Cluster No.	Organisms
2	<u>S. aureus</u> 8511 <u>S. aureus</u> 2053 <u>S. aureus</u> 2022 <u>S. aureus</u> 2031 <u>S. aureus</u> 2030 <u>S. aureus</u> 2052 <u>S. aureus</u> 187 <u>S. aureus</u> 3A <u>M. violagabriellae</u> LP <u>S. aureus</u> 6 <u>S. albus</u> 12725 <u>S. albus</u> 12761 <u>S. albus</u> 12768 <u>S. albus</u> 12897 <u>S. albus</u> 901 <u>M. violagabriellae</u> SP <u>M. violagabriellae</u> Parent <u>M. violagabriellae</u> 2a <u>S. albus</u> 12731 <u>M. tetragenus</u> <u>S. aureus</u> 2046 <u>M. halophilus</u> <u>M. candidus</u> NCIB 8610 <u>M. freudenreichii</u> NCIB 2699 <u>M. aurantiacus</u> NCIB 8609
Organisms not placed in clusters	<u>M. cyaneus</u> NCIB 9148 <u>M. cyaneus</u> CCM 856 <u>M. aurantiacus</u> NCIB 8609

DISCUSSION

The results from biochemical and physiological tests indicate that without proper sorting technique, these results are difficult to assess for the classification of Micrococci and Staphylococci. This is due to the occurrence of many different combinations of characters in this group of organisms. This variability arises mainly from the heterogeneity of the organisms, as attempts were made to minimize the variations which could be caused by using different batches of media, different growth conditions, and by non-standardization of the tests.

The results of computer analysis show that the computer-aided approach was of great assistance in sorting the data and grouping the organisms on the basis of their similarities. Discussion of the implications of the findings will be dealt with after the consideration of grouping on the basis of gas chromatography of organisms.

III

CLASSIFICATION OF STAPHYLOCOCCI AND MICROCOCCI ON THE BASIS OF CHEMICAL COMPOSITION OF ORGANISMS

LIST OF DIFFERENT COMPONENTS

Cytochromes

cyt a

cyt b

cyt c

Gas chromatograms of:

Protoplasm (Cytoplasm and Nucleoplasm)

Nucleic acids

Cell walls

Whole cells

CYTOCHROME PATTERNS

Introduction

In 1884 MacMunn was the first to observe dark lines on spectroscopic examination of myohaematin and histohaematin. However, he did not identify the pigment but demonstrated that this four-banded absorption spectrum belonged to the reduced state of the pigment and he ascribed the four distinct bands in the visible region to a single haematin compound (myohaematin or histohaematin).

In order to study this pigment, MacMunn (41) used a low dispersion microscope which sharpens the absorption bands, while the substage condenser of the microscope allows a powerful beam of light to be focused on the object under examination. This enabled him to study any opalescent or translucent material. However, other workers were using high dispersion chemical spectrometers which were suitable only for the study of the clear solutions. For the above mentioned reasons, no fundamental contribution was made until 1925 when Keilin (41) named the pigment cytochrome and stated that as well as in animals, this pigment was also found in plants and unicellular organisms, e.g. yeasts and bacteria. Furthermore, the four-banded absorption spectrum belonged not to one substance, as MacMunn believed, but to three structurally distinct haemochromagen compounds (cyt a, b, and c) which undergo reversible oxidation and reduction within living cells.

Keilin was the first to observe cytochromes of bacteria. On subjecting suspensions of Bacillus subtilis to spectroscopic examination,

he observed the same four-banded absorption spectrum occupying the following positions: cyt a - 604 m μ ; b - 564 - m μ ; c - 550 m μ ; and d - 521 - m μ . Later, both typical and somewhat modified absorption spectra of cytochromes were recorded in the cells of Bacillus subtilis; Bacillus megaterium; Proteus vulgaris; Staphylococcus aureus and Staphylococcus albus. Cytochrome patterns have not previously been used in classification schemes for bacteria, although many workers have investigated the cytochromes of particular organisms, and modern spectrophotometric methods have permitted detailed studies in some instances.

Methods and materials.

The organisms were grown on nutrient agar plates and after 24 hours of incubation at 37°C thick suspensions of organisms were made in distilled water. Before spectroscopic examination for cytochromes, suspensions were washed three times in distilled water. A small amount of sodium dithionite (approximately 1 mg sodium dithionate per ml of suspension) was added to reduce the cytochromes.

Thick opalescent suspensions were then examined in flat bottomed tubes by means of a spectroscope mounted on a microscope in place of the eyepiece. (Beck-Hartree microspectroscope) A strong source of light (carbon arc lamp) was used to illuminate the material. In some cases, where the cytochromes were difficult to see because of the masking effect of other pigments, the suspensions were allowed to stand overnight in a dilute solution of sodium dithionite. This helped to remove other pigments in the supernatant which was discarded, and the remaining suspension was examined as described above. In every instance,

the suspensions were cooled in liquid nitrogen before examination for cytochromes. Cooling to this temperature improves the resolution of the absorption bands.

Horse heart cytochrome c which was used as a reference, was obtained from Mann Research Laboratories, New York. The reference spectrum of cytochrome c and the cytochrome spectrum of the organism were examined side by side by means of a prism and auxiliary light source attached to the spectroscope. The position of the absorption bands of cytochromes was read on a wavelength scale adjusted so as to be seen with the spectroscope.

Results.

Three types of cytochromes, cyt a, b, and c were observed. The positions of these bands were as follows: cyt a - 604-605 m μ ; cyt b - 560-570 m μ ; cyt c - 550-552 m μ . Most of the Micrococci showed a three-banded (cyt a, b, c) absorption spectrum, whereas Staphylococci and Gram negative organisms showed a two-banded (cyt a, b) absorption spectrum. This is shown in Figures 1, 2, 3. The distribution of these cytochrome patterns among Staphylococci and Micrococci is recorded in Tables XII-XV. No attempt was made to distinguish between different b cytochromes.

Gas chromatography.

Gas chromatographic analysis was performed on 16 different organisms (Staphylococci, Micrococci, Gram negative organisms). Whole cells and other components (protoplasm, nucleic acids, cell walls) of

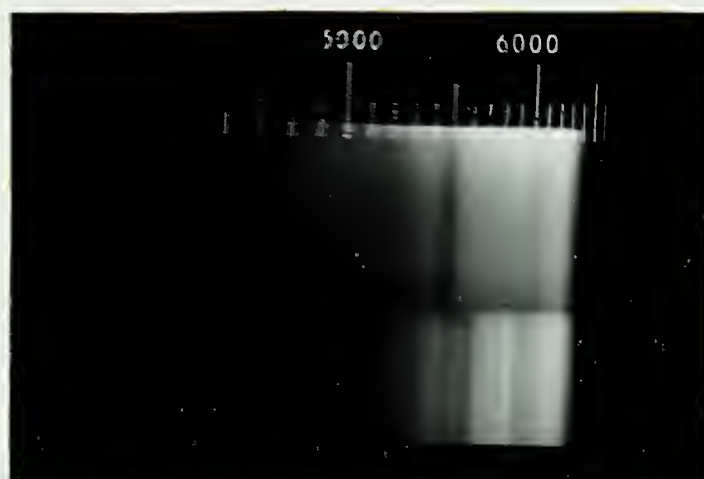


Fig. 1. Visual spectroscopy: cytochrome pattern of typical Micrococci with reference horse heart cyt c on the upper spectrum (550 mμ).



Fig. 2. Visual spectroscopy: cytochrome pattern of typical Staphylococci with reference horse heart cyt c on the upper spectrum.

NOTE: A slight correction should be made, as the sodium line in the Micrococci spectrum (Fig. 1) is slightly displaced to the left - it should have been in line with the reference sodium line on the upper scale.

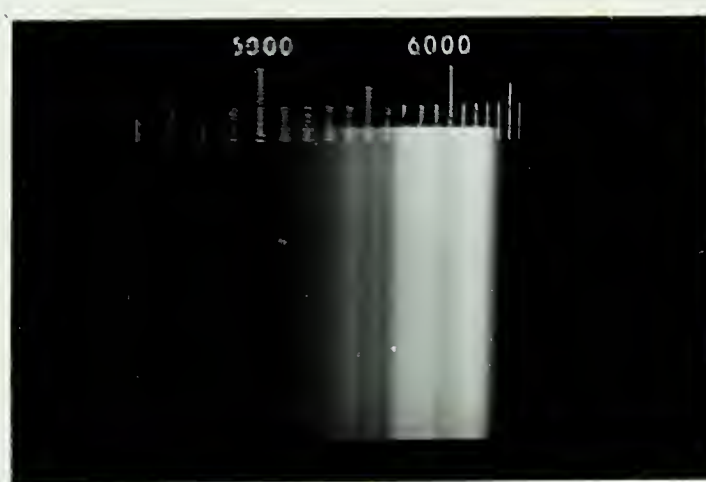


Fig. 3. Visual spectroscopy: cytochrome pattern of typical Micrococci without reference horse heart cyt c.

TABLE XII

CYTOCHROME PATTERNS: VISUAL SPECTROSCOPY OF
MISCELLANEOUS STRAINS OF M. LUTEUS

Organisms	cyt. c	cyt. b	cyt. a
<u>M. luteus</u> NCIB 8165	+	+	+
CCM 853	+	+	+
" 559	+	+	+
" 622	+	+	+
" 1335	+	+	+
" 852	+	+	+
" 309	+	+	+
" 247	+	+	+
" 855	+	+	+
" 851	+	+	+
" 248	+	+	+
" 210	+	+	+
" 266	+	+	+
" 265	+	+	+
" 331	+	+	+

TABLE XIII

CYTOCHROME PATTERNS: VISUAL SPECTROSCOPY
OF MISCELLANEOUS STRAINS OF MICROCOCCI

Organism		cyt. c	cyt. b.	cyt. a
<u>M. conglomeratus</u>	CCM 208	+	+	+
	CCM 2677	+	+	+
<u>M. varians</u>	CCM 1046	+	+	+
	CCM 250	+	+	+
	CCM 884	+	+	+
<u>M. roseus</u>	NCIB 8175	+	+	+
	NCIB 146	+	+	+
	CCM 706	+	+	+
<u>M. cyaneus</u>	NCIB 9148	-	+	+
	CCM 856	-	+	+
<u>M. candidus</u>	NCIB 8610	-	+	+
<u>M. freudenreichii</u>	NCIB 2699	-	+	+
<u>M. aurantiacus</u>	NCIB 8609	-	+	+
<u>M. flavus</u>	NCIB 8166	+	+	+
<u>M. lysodeikticus</u>	NCIB 9278	+	+	+
<u>M. sodonensis</u>	NCIB 8854	+	+	+
<u>M. cereus</u>		+	+	+
<u>M. saprophiticus</u>		+	+	+
<u>M. halophilus</u>		-	+	+
<u>M. tetragenus</u>		-	+	+
<u>M. violagabriellae</u>	2a	-	+	+
	parent	-	+	+
	super pig	-	+	+
	little pig	-	+	+

TABLE XIV

CYTOCHROME PATTERNS: VISUAL SPECTROSCOPY OF
MISCELLANEOUS STRAINS OF STAPHYLOCOCCI

Organisms	cyt. c	cyt. b	cyt. a
<u>S. aureus</u> strains			
2022	-	+	+
8511	-	+	+
2053	-	+	+
2051	-	+	+
2061	-	+	+
2052	-	+	+
2046	-	+	+
2030	-	+	+
2057	-	+	+
2031	-	+	+
2024	-	+	+
2034	-	+	+
3A	-	+	+
6	-	+	+
187	-	+	+
52	-	+	+
42D	-	+	+
29	-	+	+
<u>S. albus</u> strains			
12897	-	+	+
12761	-	+	+
12768	-	+	+
12731	-	+	+
12725	-	+	+

TABLE XV

CYTOCHROME PATTERNS: VISUAL SPECTROSCOPY OF
MISCELLANEOUS GRAM NEGATIVE ORGANISMS

Organisms	cyt. c	cyt. b	cyt. a
11339	-	+	+
11336	-	+	+
11330	-	+	+
11318	-	+	+
11305	-	+	+
11337	-	+	+
11334	-	+	+
11344	-	+	+
11319 pyo.	+	+	+
11319 pky. wh.	+	+	+
12036	-	+	+
11933	-	+	+
11947	-	+	+
11948	-	+	+
11854	-	+	+
11787	-	+	+
11852	-	+	+
11851	-	+	+
PMU 218	-	+	+
" 223	-	+	+
12096	-	+	+
11949	-	+	+
11946	-	+	+
12100	-	+	+
12255	-	+	+
12331	-	+	+
12265	-	+	+
12389	-	+	+
12324	-	+	+
12356	-	+	+
12065	-	+	+
12332	-	+	+
12465	-	+	+
12493	-	+	+
12590	-	+	+
12403	-	+	+

(Contd.)

TABLE XV (Contd.)

Organisms	cyt. c.	cyt. b	cyt. a
12462	-	+	+
12516	-	+	+
12566	-	+	+
12503	-	+	+
12501	-	+	+
12448	-	+	+
12450	-	+	+
12591	-	+	+
12594	-	+	+
12447	-	+	+
15767	-	+	+
12616	-	+	+
12613	-	+	+
15796	-	+	+
12558	-	+	+
15751	-	+	+

these organisms were prepared as follows:

Preparation of whole cells.

1. The organisms were streaked on NYA plates and harvested after 36 hours of incubation at 37°C. The harvested cells were washed three times in distilled water by centrifugation and re-suspended in water. Finally, they were lyophilized in order to be used for pyrolysis.

2. The organisms were grown in nutrient broth (Oxoid #2) + .5% yeast extract in 1-liter jars with constant stirring at 37°C. The Staphylococci were harvested after 24 hours by centrifugation at 10,000 RPM in a Sorvall refrigerated centrifuge. The harvested cells were washed three times in distilled water by centrifugation and re-suspended in distilled water to a concentration of about 200 mg wet cells per ml. One ml of this suspension was lyophilized for analysis. Micrococci required a longer time for growth, and were therefore harvested after 36 hours of incubation.

Preparation of protoplasm

The suspensions of cells from No. 2 were broken down under pressure by means of a Sorvall-Ribi Refrigerated Cell Fractionator. In order to avoid denaturation of proteins, the cells were chilled in ice before processing and the Ribi valve was also pre-cooled to -5°C. Gram negative organisms were subjected to a pressure of 10,000 - 20,000 psi, whereas Staphylococci and Micrococci required a pressure of 50,000 - 60,000 psi. In order to ensure a maximum breakage, the suspensions were run through the Ribi Fractionator twice. The effluents were

collected in flasks immersed in ice/water mixture and centrifuged at 15,000 r.p.m. (27,000 x G) in a Sorvall Refrigerated Centrifuge using an SS - 34 rotor. After two hours of centrifugation, the supernatant containing soluble protoplasm, and the residue which was predominantly of cell walls and a few unbroken cells were separated from each other. A portion of the supernatant was freeze-dried for gas chromatograph analysis and the rest was used for the extraction of nucleic acids.

Preparation of cell walls.

The crude cell walls obtained as a residue by the above procedure were washed three times by suspension in 1M sodium chloride, centrifuged at 12,000 r.p.m. (17,300 x G) for 90 minutes and then washed in distilled water at 8,000 r.p.m. (7100 x G) until chloride free. The presence or absence of chloride was checked by adding 1 ml of silver nitrate solution to 1 ml of the supernatant. The appearance of white precipitate indicated the presence of chloride in the supernatant, in which case further washings were done until no precipitate appeared.

Preparation of nucleic acids.

To 3 ml of the supernatant containing protoplasm, one volume of 40% streptomycin sulphate solution was added to precipitate nucleic acids (49). The precipitate was collected by centrifugation in a clinical centrifuge and then washed in water at 3800 r.p.m. (1700 G) until streptomycin sulphate free. The presence or absence of streptomycin sulphate was detected by adding 2 ml of absolute ethyl alcohol to 1 ml of the supernatant obtained after each washing. The appearance of white precipitate indicated the presence of streptomycin sulphate, in

which case further washings were done until no precipitate appeared on addition of ethyl alcohol to the supernatant. The precipitate thus obtained was dried in watch glasses and preserved in screw-cap bottles for gas chromatographic analysis.

Gas chromatographic analysis.

The analysis was performed on 16 different organisms, the whole cells (grown on nutrient agar, nutrient broth) and their components (protoplasm, cell walls, nucleic acids). A Carlo Erba Fractovap Model GV gas chromatograph equipped with a pyrolysis cell was used for this purpose. The sample (approximately 1 mg) to be analyzed was placed inside the nickel chrome filament with a mica sheet (5 - 6 mm²) at one end of the spiral to prevent the sample falling. The sample was pyrolyzed at 850°C for a standard time (10 seconds). The thermally degraded products were detected by using a dual column operation. The columns consisted of stainless steel tubing 6 mm in outside diameter and 2 meters long, and were packed with chromosorb W 60/80 mesh coated with 10% 20 M carbowax. The analysis was done over a programmed temperature range of 60° - 180° with an increase of 10° per minute. The linear heating program was combined with initial isothermal period of 5 minutes at 60° and final isothermal period of 8 minutes at 180°. The carrier gas was nitrogen and the flow rate 40 ml per minute. Two hydrogen flame ionization detectors were used for differential analysis. Whole cells, protoplasm, nucleic acids, and a few samples of cell walls were pyrolyzed by the above method. Cell wall analysis could not be completed in the time available because of excessive 'bleeding' and exhaustion of

the column material in the last stage of the operation.

Results.

Chromatograms obtained after pyrolysis of whole cells and their components are shown in Figures 4 - 19. Since it is inconvenient to include all the gas chromatograms, a few representatives of each group were chosen and the gas chromatograms of their whole cells and components have been reproduced in Figure 4-19. For the Micrococcus group of organisms, Micrococcus luteus CCM 248 was chosen and for the Staphylococcus group, Staphylococcus aureus 29, Staphylococcus aureus 2057, and Staphylococcus albus 12731 were chosen. E. coli 11336 was used for comparison purposes. A few organisms were chosen at random to show the effects of different kinds and different brands of growth media and the effect of change of column material on the reproducibility of these chromatograms from one run to another.

In an attempt to evaluate the possible taxonomic use of the data, chromatograms were analysed by procedures of numerical taxonomy.

Analysis of chromatograms with the aid of a computer.

Chromatograms of whole cells grown on NYA nutrient broth (+ 0.5% yeast extract) and their components (nucleic acids and cytoplasm) were analyzed by computer analysis. The program developed by George (29) et al., which is described in Part I, was utilized for instructing the computer. The analysis was done on the basis of the individual peaks on the gas chromatogram. Each peak in these chromatograms was looked upon as an attribute. Hence the total number of peaks in a given

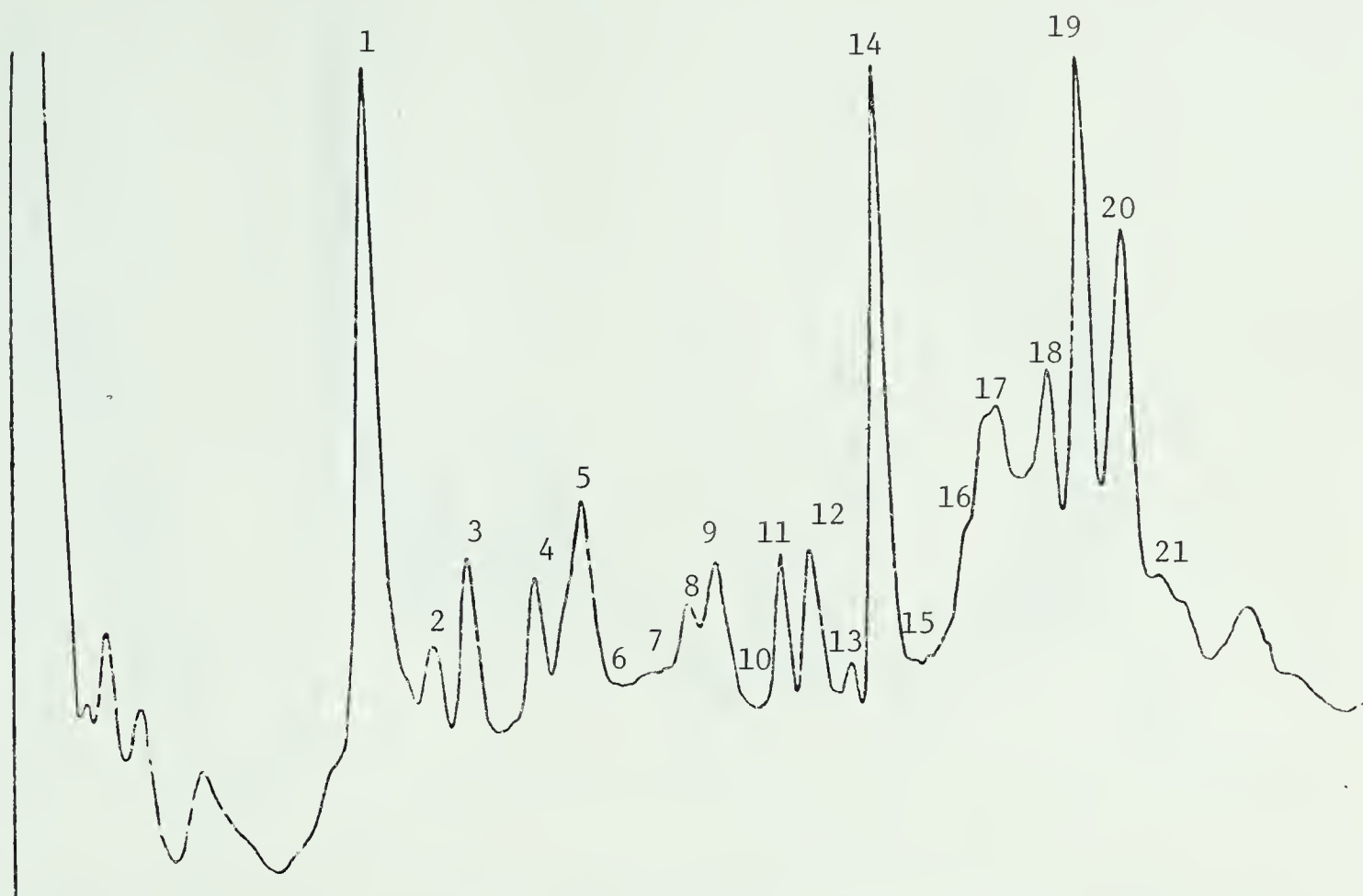


Fig.4 Gas chromatogram of whole cells of Staphylococcus aureus 2057 grown on NYB medium.

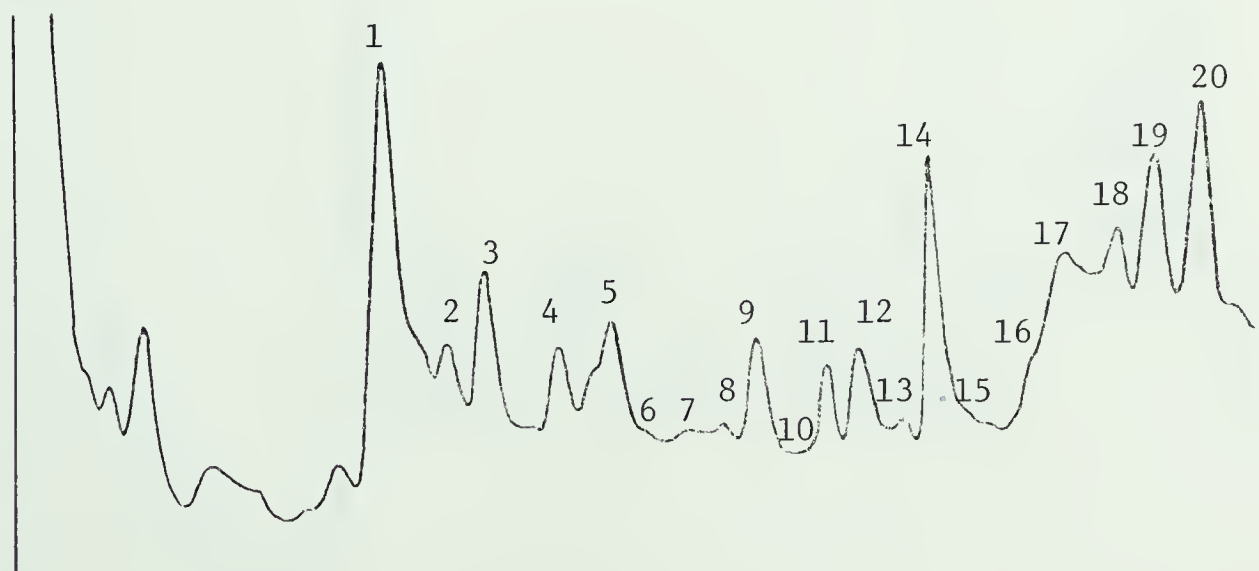


Fig.5. Gas chromatogram of whole cells of Micrococcus sodonensis grown on NYB medium.

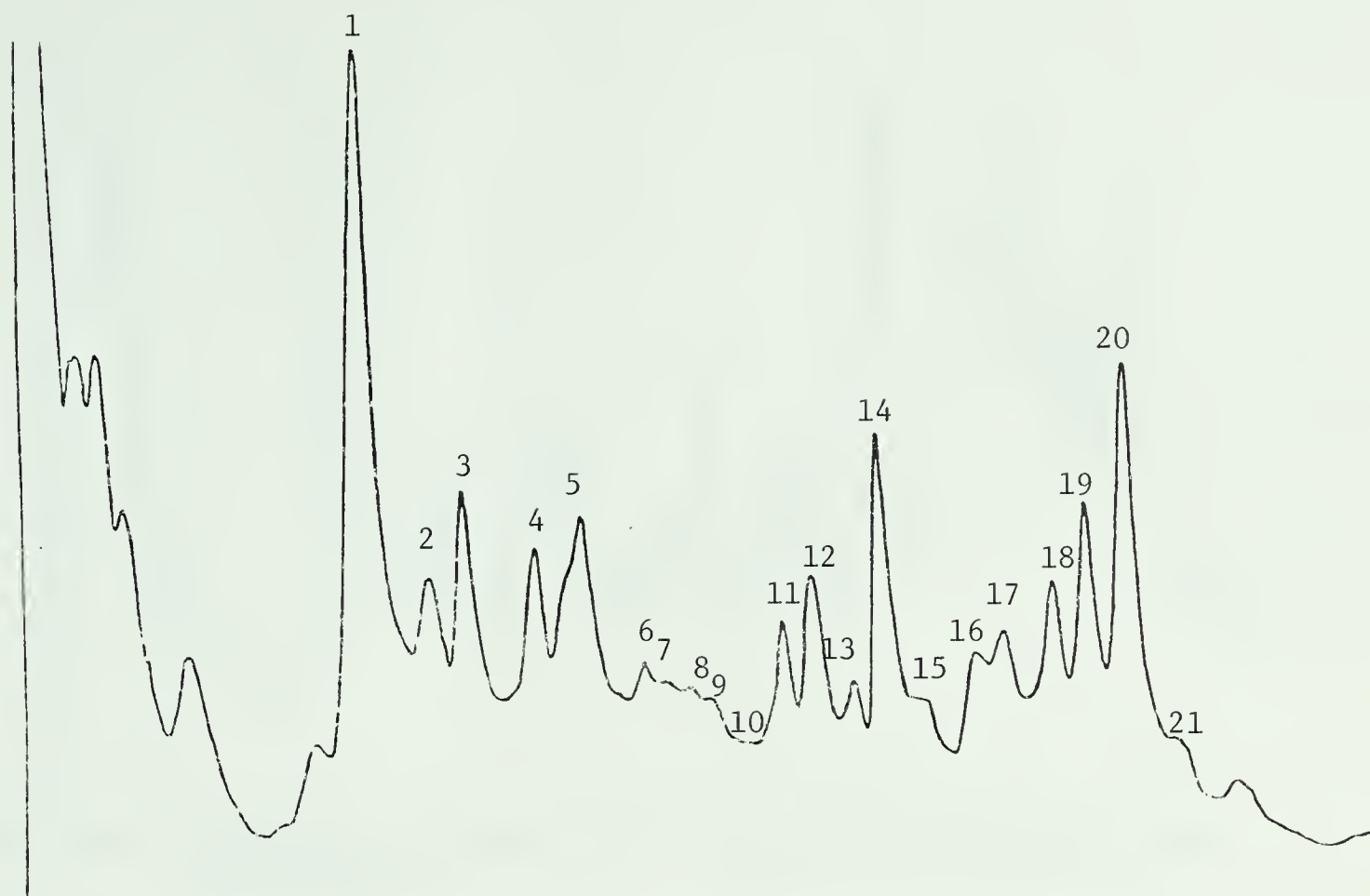


Fig.6. Gas chromatogram of whole cells of *Staphylococcus albus* 12731 grown on NYA medium.

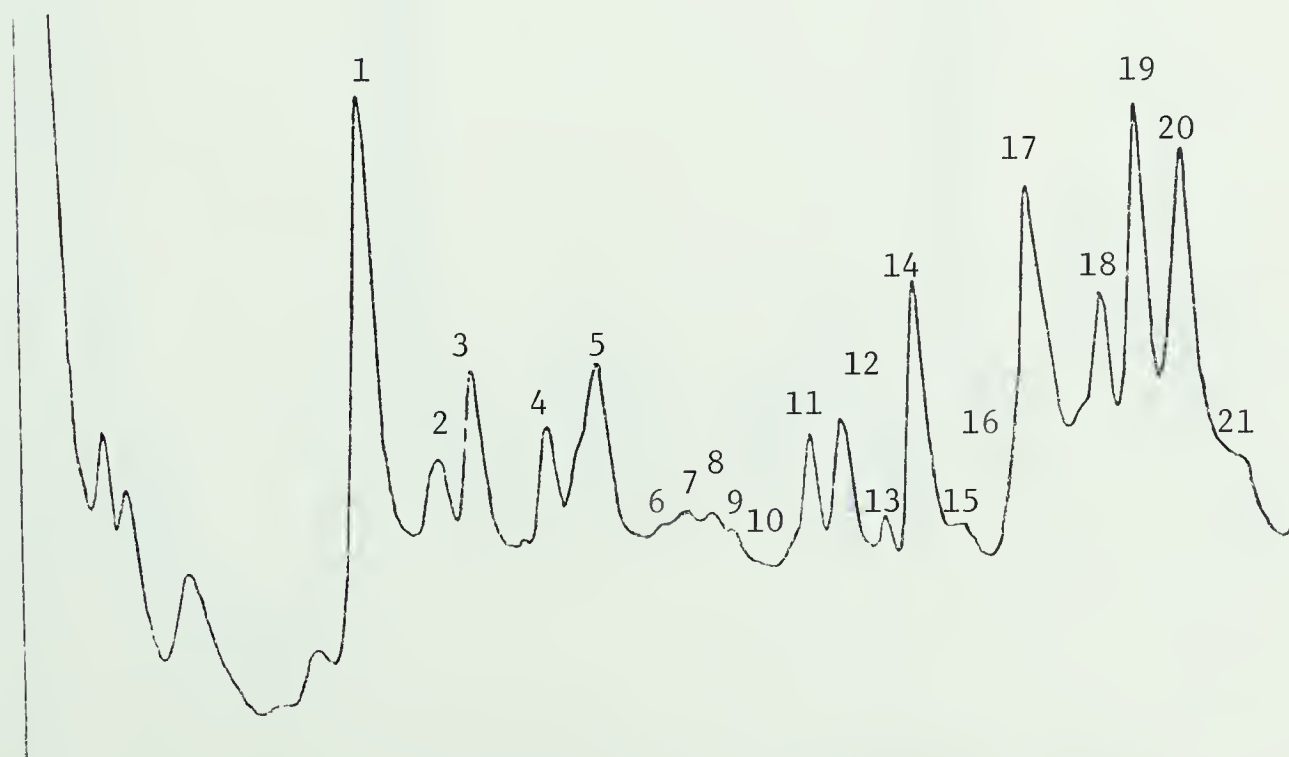


Fig.7. Gas chromatogram of whole cells of *Staphylococcus albus* 12731 grown on NYB medium.

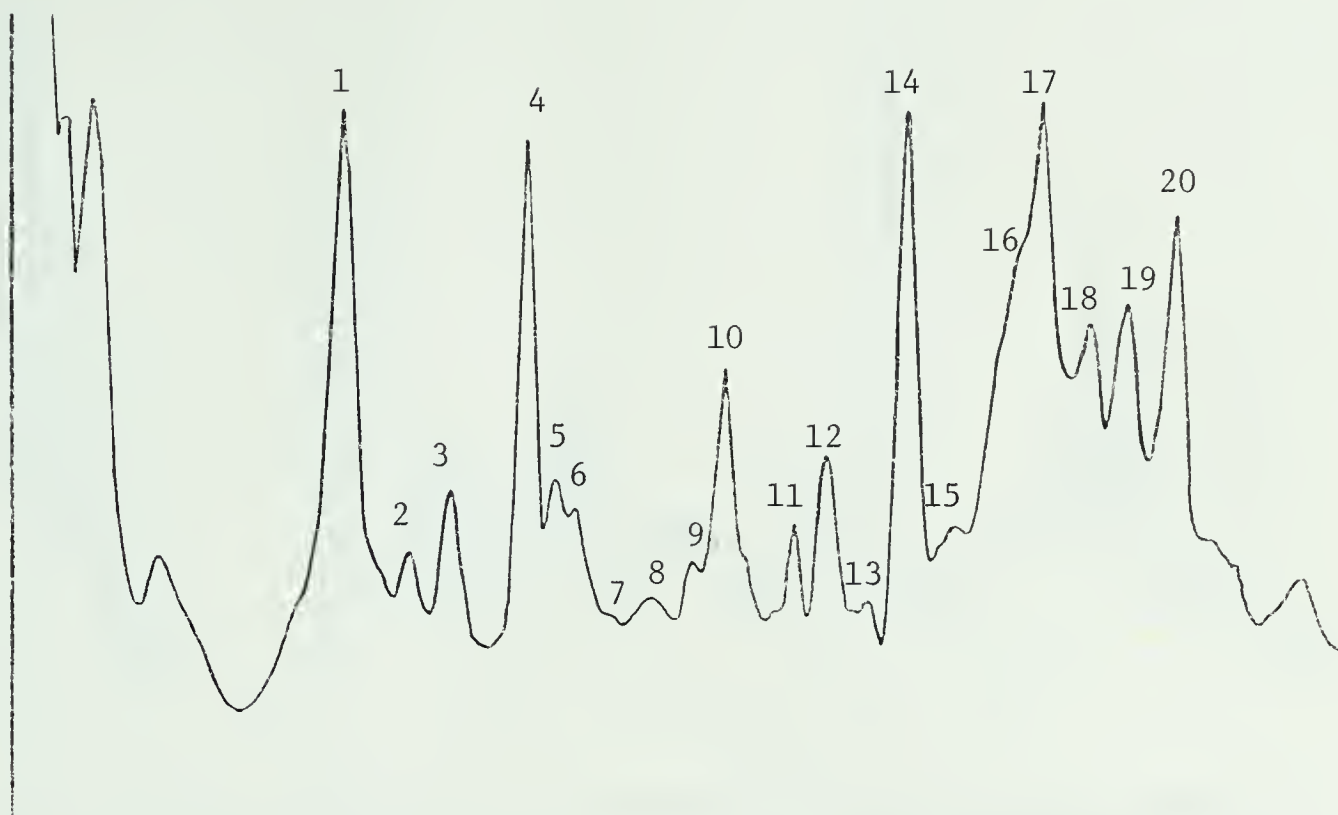


Fig.8. Gas chromatogram of whole cells of Micrococcus halophilus grown on NYA medium.

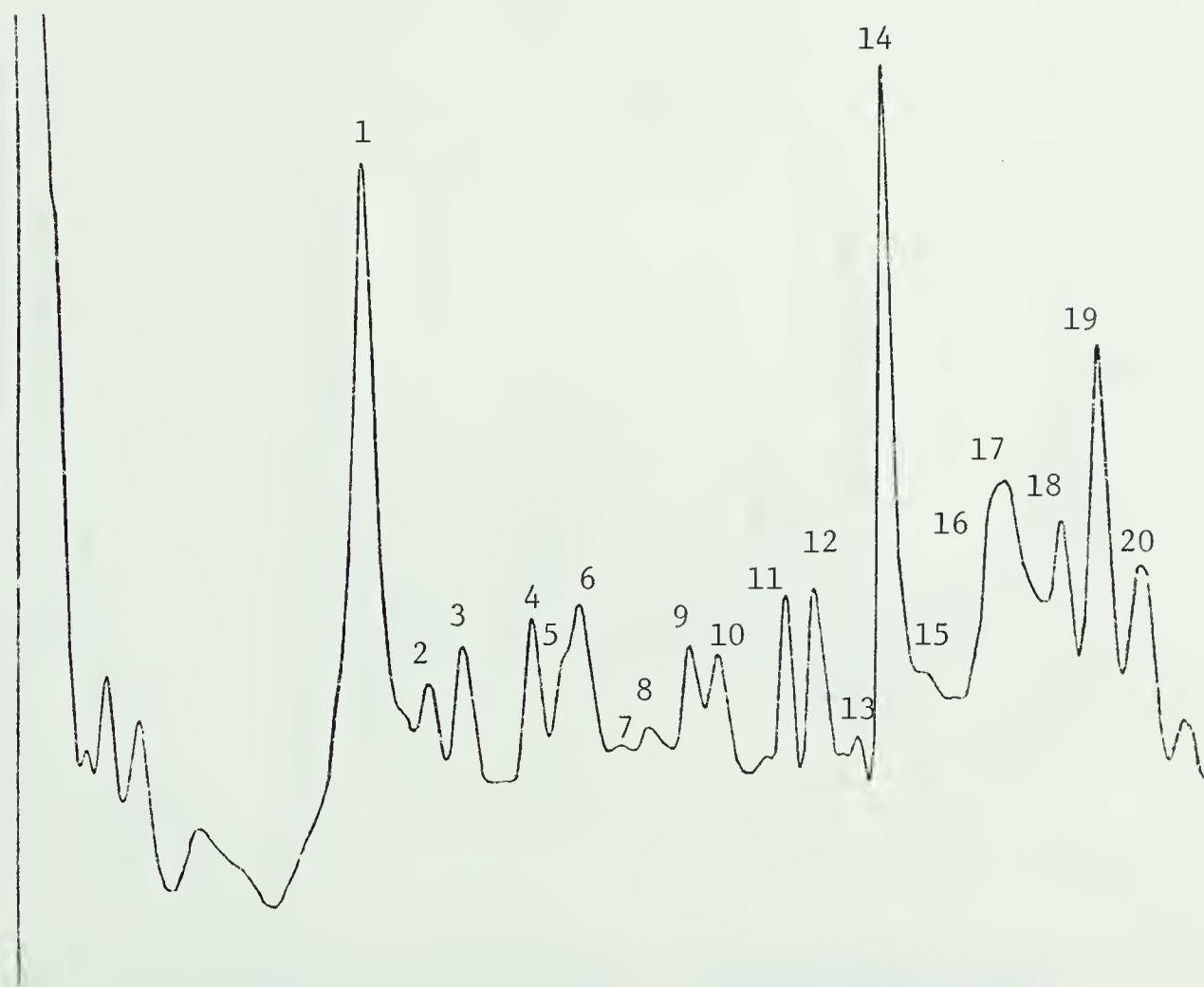


Fig.9. Gas chromatogram of whole cells of Micrococcus halophilus grown on NYB medium.

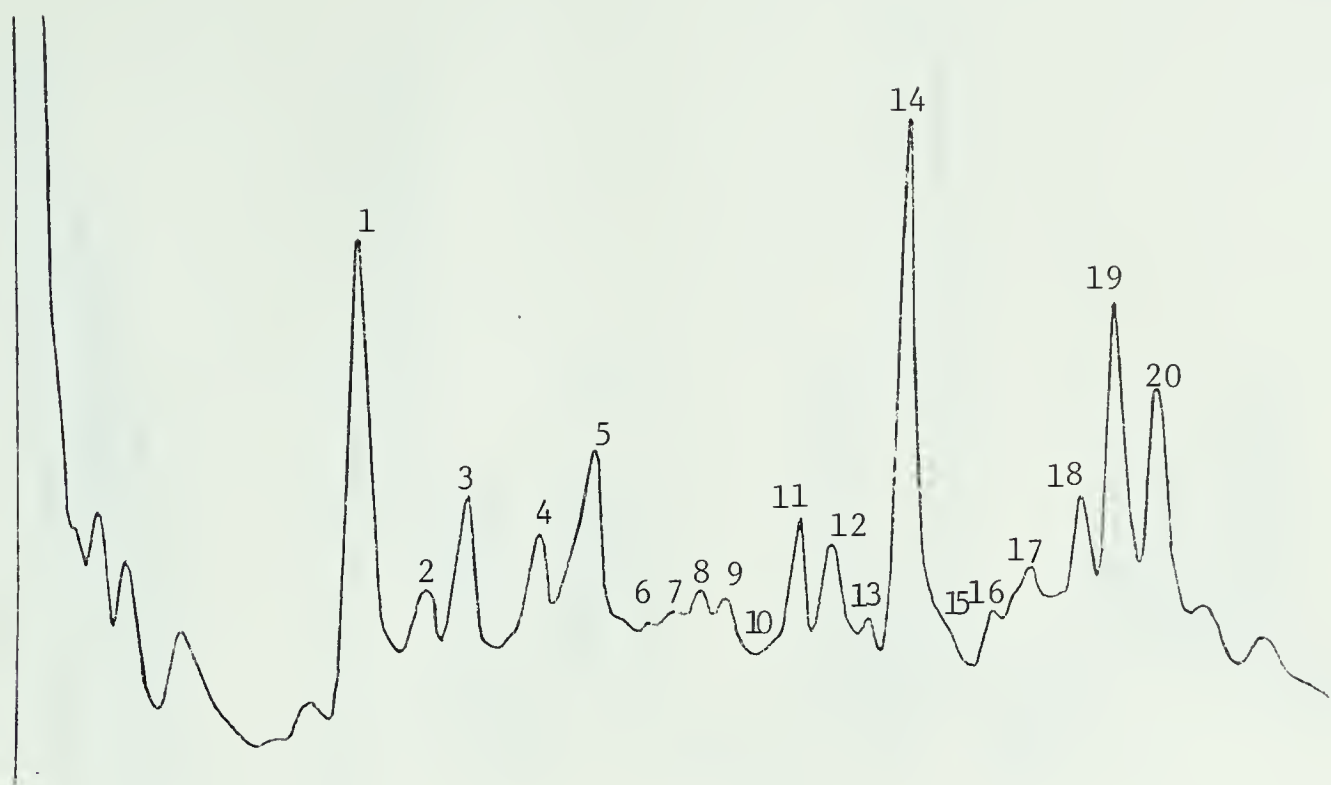


Fig. 10. Gas chromatogram of protoplasm of Staphylococcus aureus 29.

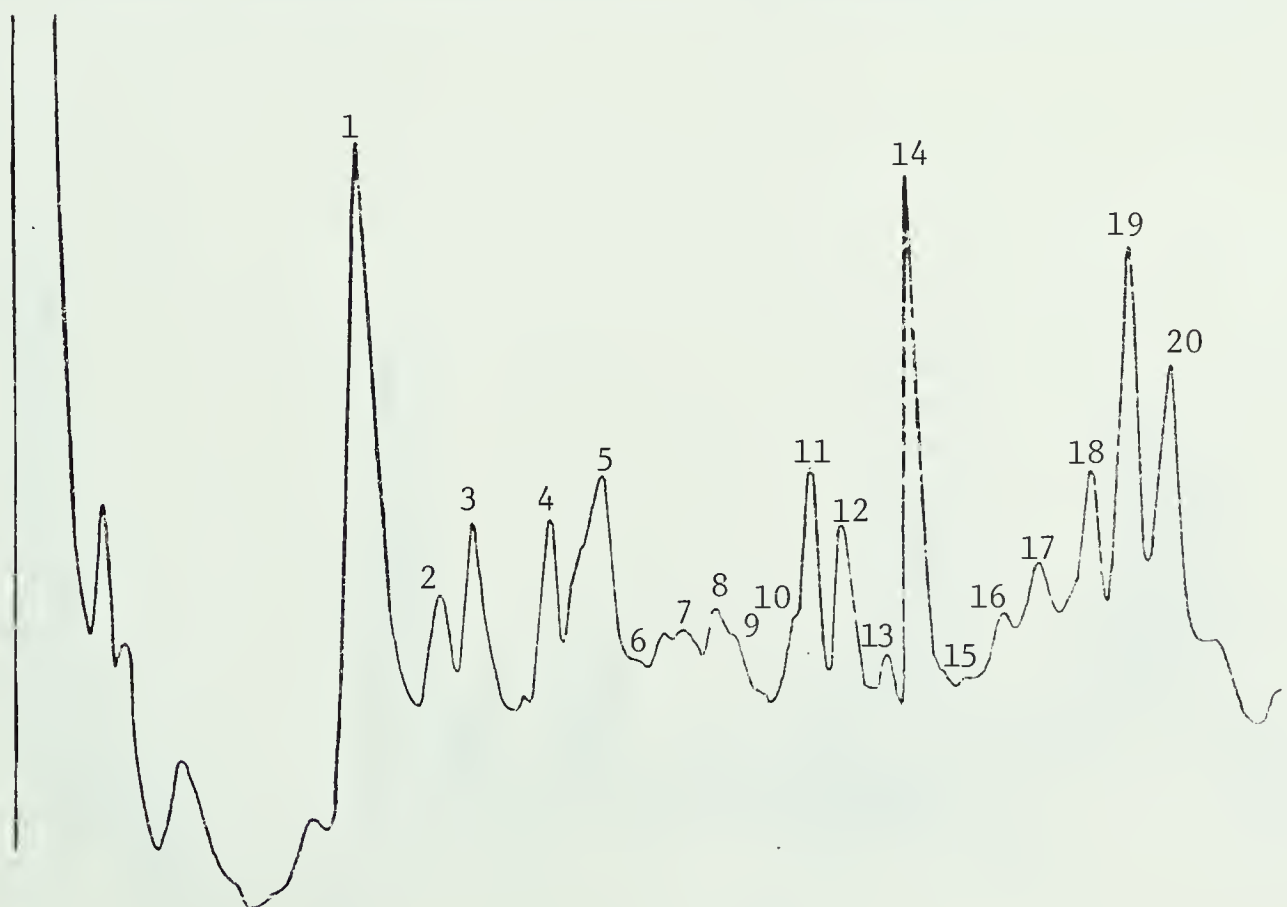


Fig. 11. Gas chromatogram of protoplasm of Staphylococcus albus 12731.

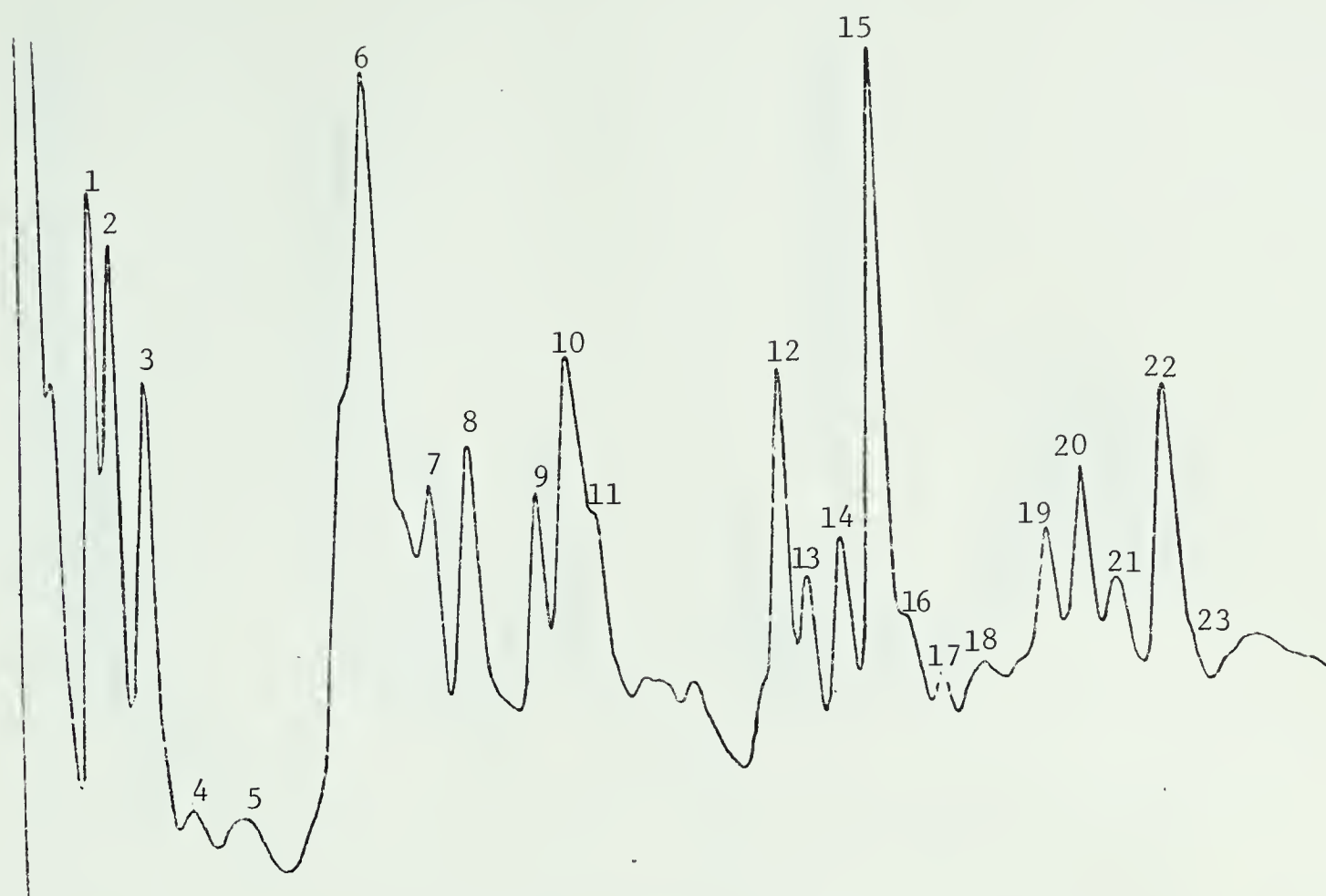


Fig. 12. Gas chromatogram of nucleic acids of Staphylococcus aureus 29.

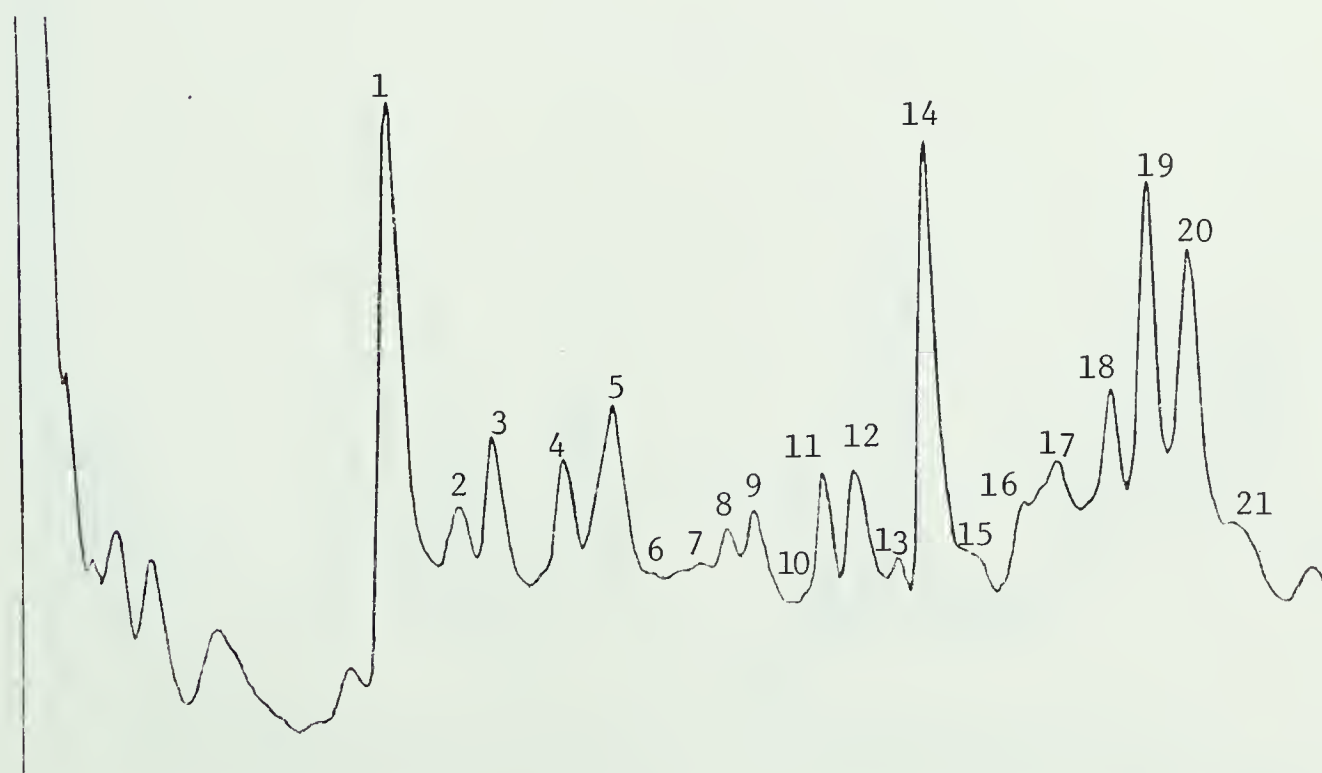


Fig. 13. Gas chromatogram of whole cells of Staphylococcus aureus grown on NYB medium.

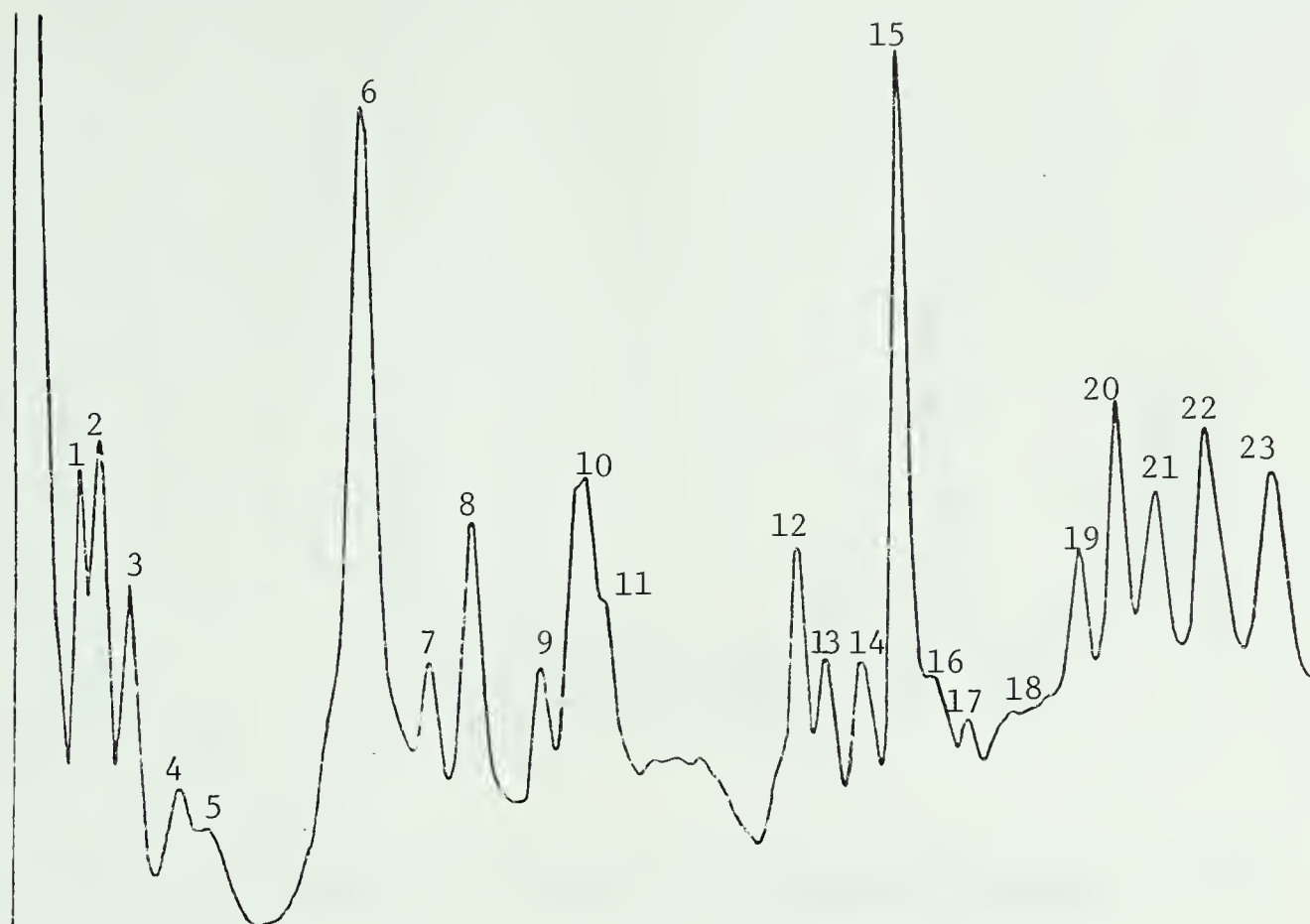


Fig. 14. Gas Chromatogram of nucleic acids of Micrococcus halophilus.

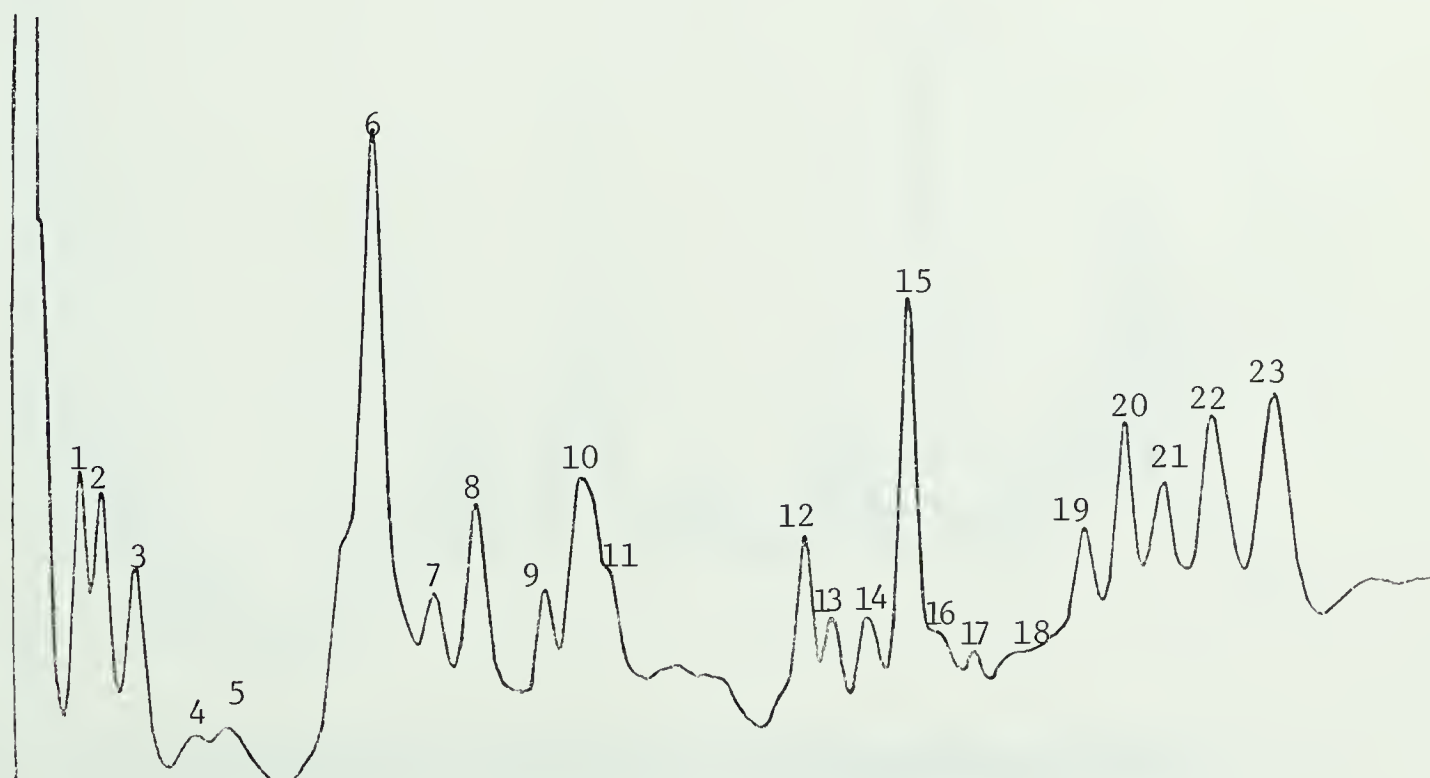


Fig. 15. Gas chromatogram of nucleic acid of Staphylococcus aureus 2057.

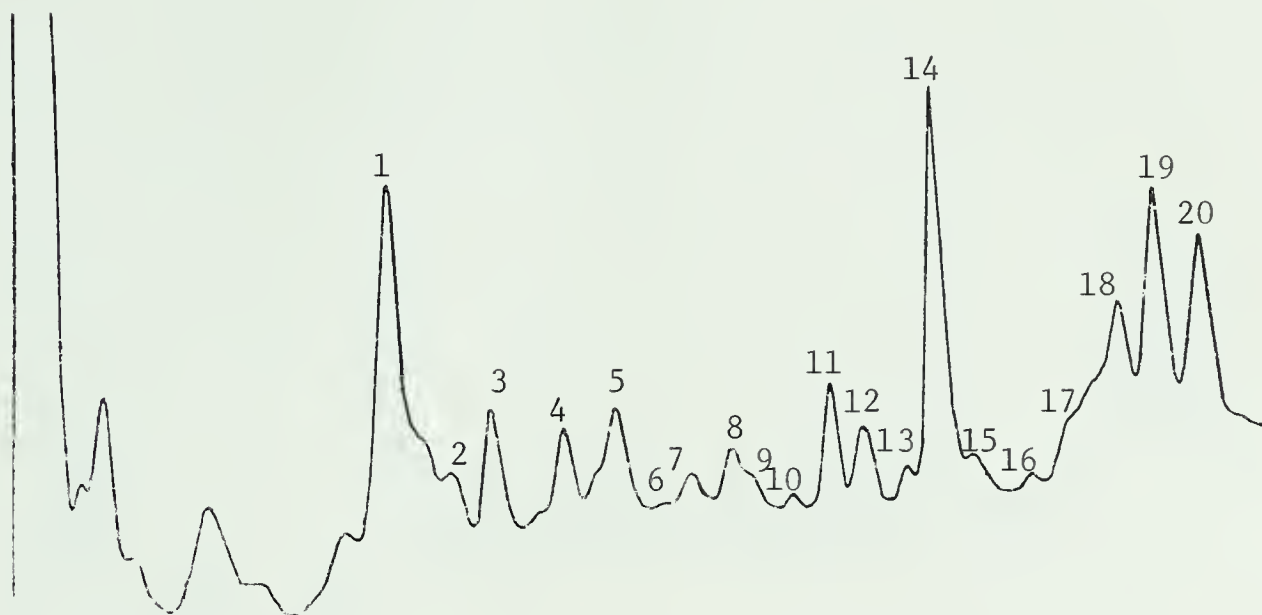


Fig. 16. Gas chromatogram of protoplasm of Micrococcus luteus CCM 248.

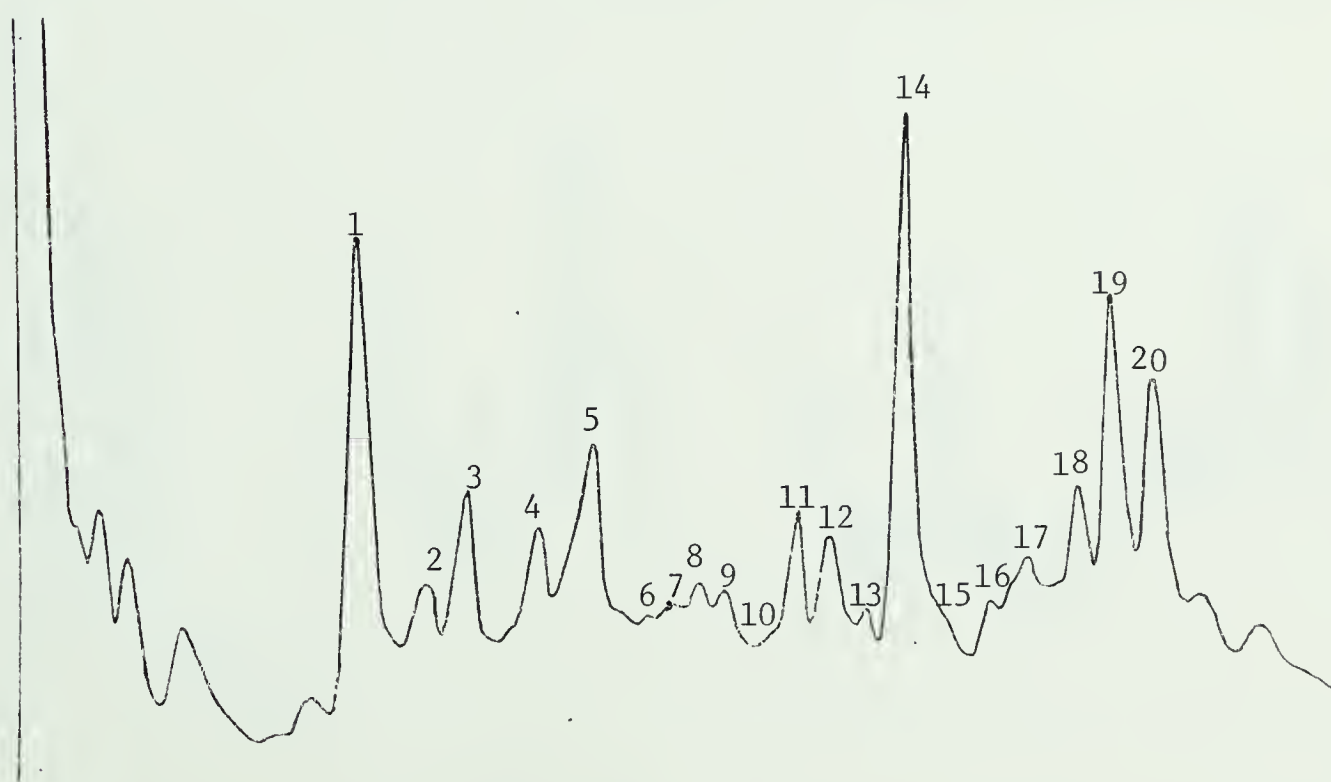


Fig. 17. Gas chromatogram of protoplasm of Staphylococcus aureus 29.

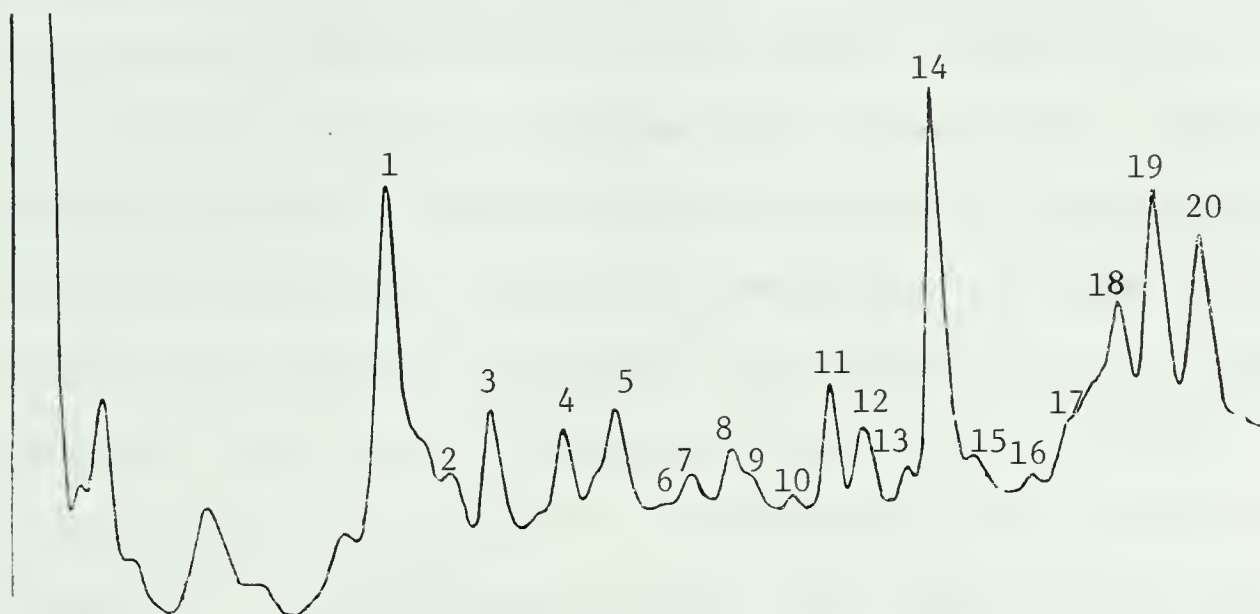


Fig. 18. Gas chromatogram of protoplasm of *Micrococcus luteus* CCM 248.

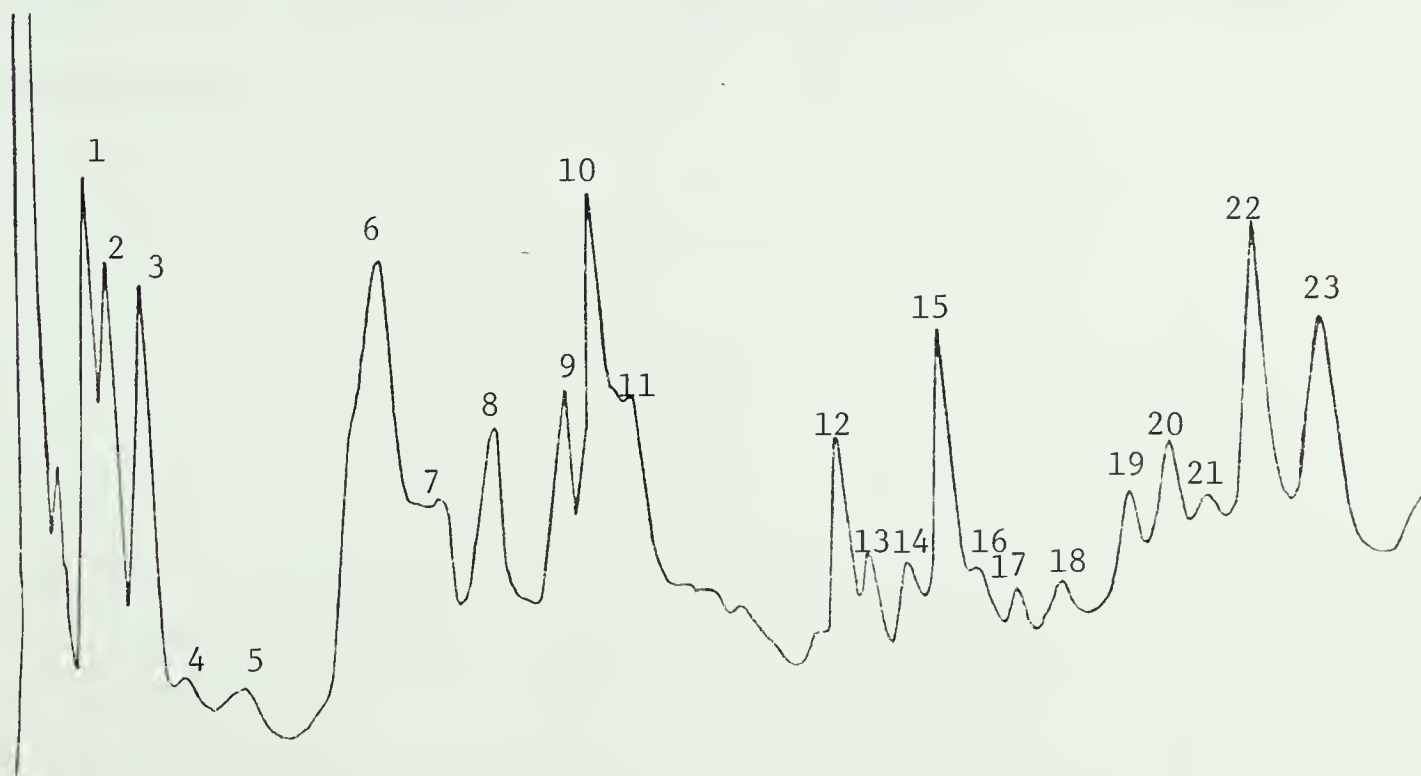


Fig. 20. Gas chromatogram of nucleic acids of *Micrococcus luteus* CCM 248.

chromatogram corresponded to the total number of attributes for the sample under study. For a given set of samples of nucleic acids, whole cells, or protoplasm, the total number of these attributes was kept constant for all the organisms under consideration. Therefore a chromatogram with the maximum number of peaks and troughs was used as a key for labelling all the other chromatograms. The peak and trough heights were measured at 42 places on the chromatogram. The measurements were taken from the base line drawn by joining the lowest trough to the last trough on the chromatogram. This is shown in Figure 20. The measurements obtained were entered in the program as if they were individual characteristics. The values thus obtained were entered in the program as if they were individual characteristics. The computer analysis results of gas chromatograms of whole cells (grown on NYA, NYB medium), and the cell components (nucleic acids, protoplasms) are shown in Tables XVI - XXIX.

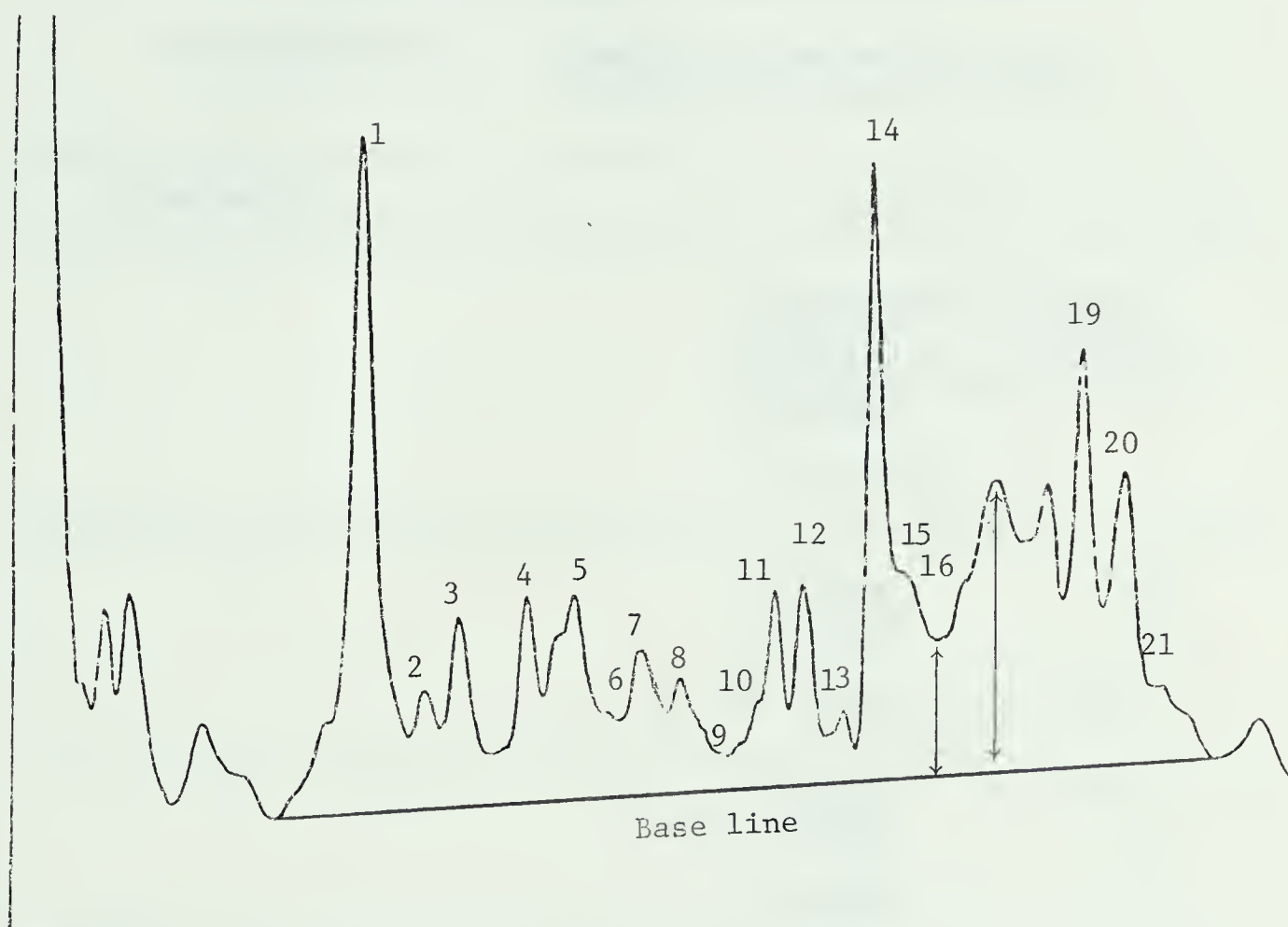


Fig. 20. Technique of measuring chromatograms for computer analysis.

TABLE XVI

RESULTS FROM COMPUTER ANALYSIS OF GAS CHROMATOGRAMS OF
WHOLE CELLS GROWN ON NUTRIENT AGARRESOLUTION LEVEL 1 Average link parameter 0.770
Single link parameter 0.770

Cluster No.	Organism
1	<u>M. sodonensis</u> NCIB 8854 <u>M. luteus</u> NCIB 8165 <u>M. lysodeikticus</u> NCIB 9278 <u>M. cereus</u> <u>M. tetragenus</u>
2	<u>S. aureus</u> 29 <u>S. aureus</u> 6 <u>M. candidus</u> NCIB 8610
3	<u>S. aureus</u> 2031 <u>S. aureus</u> 3A <u>S. aureus</u> 187
Satellite of Cluster 2	<u>M. candidus</u> NCIB 8610
Satellite of Cluster 3	<u>S. aureus</u> 187
Satellite of Cluster 3	<u>M. roseus</u> NCIB 8175
Satellite of Cluster 2	<u>M. tetragenus</u>
Organisms not placed in clusters	<u>M. halophilus</u> <u>S. albus</u> 12731

TABLE XVII

RESULTS FROM COMPUTER ANALYSIS OF GAS CHROMATOGRAMS
OF WHOLE CELLS GROWN ON NUTRIENT AGAR

RESOLUTION LEVEL 2 Average link parameter 0.739
Single link parameter 0.755

Cluster No.	Organisms
1	<u>M. sodonensis</u> NCIB 8854 <u>M. luteus</u> NCIB 8165 <u>M. lysodeikticus</u> NCIB 9278 <u>M. cereus</u> <u>M. tetragenus</u>
2	<u>S. aureus</u> 29 <u>S. aureus</u> 6 <u>M. candidus</u> NCIB 8610 <u>M. tetragenus</u>
3	<u>S. aureus</u> 2031 <u>S. aureus</u> 3A <u>S. aureus</u> 187 <u>M. roseus</u> NCIB 8175
Satellite of Cluster 3	<u>M. roseus</u> NCIB 8175
Satellite of Cluster 2	<u>M. tetragenus</u>
Organisms not placed in clusters	<u>M. halophilus</u> <u>S. albus</u> 12731

TABLE XVIII

RESULTS FROM COMPUTER ANALYSIS OF GAS CHROMATOGRAMS
OF WHOLE CELLS GROWN ON NUTRIENT AGAR

RESOLUTION LEVEL 3 Average link parameter 0.708
 Single link parameter 0.739

RESOLUTION LEVEL 4* Average link parameter 0.677
 Single link parameter 0.724

Cluster No.	Organisms
1	<u>M. sodonensis</u> NCIB 8854 <u>M. luteus</u> NCIB 8165 <u>M. lysodeikticus</u> NCIB 9278 <u>M. cereus</u> <u>M. tetragenus</u>
2	<u>S. aureus</u> 29 <u>S. aureus</u> 6 <u>M. candidus</u> NCIB 8610 <u>M. tetragenus</u>
3	<u>S. aureus</u> 2031 <u>S. aureus</u> 3A <u>M. roseus</u> NCIB 8175 <u>M. halophilus</u>
Satellite of Cluster 2	<u>M. tetragenus</u>
Organisms not placed in clusters	<u>M. halophilus</u> <u>S. albus</u> 12731

* Tables corresponding to resolution levels 3 and 4
 are exactly similar.

TABLE XIX

RESULTS FROM COMPUTER ANALYSIS OF GAS CHROMATOGRAMS OF
WHOLE CELLS GROWN ON NUTRIENT AGAR

RESOLUTION LEVEL 5 Average link parameter 0.647
 Single link parameter 0.708

RESOLUTION LEVEL 6* Average link parameter 0.616
 Single link parameter 0.693

Cluster No.	Organisms
1	<u>M. sodonensis</u> NCIB 8854 <u>M. luteus</u> NCIB 8165 <u>M. lysodeikticus</u> NCIB 9278 <u>M. cereus</u> <u>M. tetragenus</u>
2	<u>S. aureus</u> 29 <u>S. aureus</u> 6 <u>M. candidus</u> NCIB 8610 <u>M. tetragenus</u> <u>M. cereus</u>
3	<u>S. aureus</u> 2031 <u>S. aureus</u> 3A <u>S. aureus</u> 187 <u>M. roseus</u> NCIB 8175 <u>M. halophilus</u>
Satellite of Cluster 2	<u>M. tetragenus</u>
Organisms not placed in clusters	<u>M. halophilus</u> <u>S. albus</u> 12731

* Tables corresponding to resolution levels 5 and 6
 are exactly similar.

TABLE XX

RESULTS FROM COMPUTER ANALYSIS OF GAS CHROMATOGRAMS
OF WHOLE CELLS GROWN ON NUTRIENT AGAR

RESOLUTION LEVEL 7 Average link parameter 0.585
Single link parameter 0.677

Cluster No.	Organisms	
1	<u>M. sodonensis</u>	NCIB 8854
	<u>M. luteus</u>	NCIB 8165
	<u>M. lysodeikticus</u>	NCIB 9278
	<u>M. cereus</u>	
	<u>M. tetragenus</u>	
	<u>S. aureus</u>	29
	<u>S. aureus</u>	6
	<u>M. candidus</u>	NCIB 8610
	<u>S. albus</u>	12731
	<u>M. halophilus</u>	
2	<u>S. aureus</u>	2031
	<u>S. aureus</u>	3A
	<u>S. aureus</u>	187
	<u>M. roseus</u>	NCIB 8175
	<u>M. halophilus</u>	
Organism not placed in clusters	<u>M. halophilus</u>	

TABLE XXI

RESULTS FROM COMPUTER ANALYSIS OF GAS CHROMATOGRAMS
OF WHOLE CELLS GROWN ON NUTRIENT AGAR

RESOLUTION LEVEL 8	Average link parameter 0.554
	Single link parameter 0.662
RESOLUTION LEVEL 9*	Average link parameter 0.523
	Single link parameter 0.647
RESOLUTION LEVEL 10*	Average link parameter 0.492
	Single link parameter 0.631
RESOLUTION LEVEL 11*	Average link parameter 0.462
	Single link parameter 0.616
RESOLUTION LEVEL 12*	Average link parameter 0.431
	Single link parameter 0.600

Cluster No.	Organisms	
1	<u>M. sodonensis</u>	NCIB 8854
	<u>M. luteus</u>	NCIB 8165
	<u>M. lysodeikticus</u>	NCIB 9278
	<u>M. cereus</u>	
	<u>M. tetragenus</u>	
	<u>S. aureus</u>	29
	<u>S. aureus</u>	6
	<u>M. candidus</u>	NCIB 8610
	<u>S. albus</u>	12731
	<u>M. halophilus</u>	
	<u>S. aureus</u>	187
2	<u>S. aureus</u>	2031
	<u>S. aureus</u>	3A
	<u>S. aureus</u>	187
	<u>M. roseus</u>	NCIB 8175
	<u>M. halophilus</u>	

* Tables corresponding to resolution levels 9, 10, 11 and 12 are exactly similar to table for resolution level 8.

TABLE XXII

RESULTS FROM COMPUTER ANALYSIS OF GAS CHROMATOGRAMS
OF WHOLE CELLS GROWN ON NUTRIENT BROTH

RESOLUTION LEVEL 1 Average link parameter 0.770
 Single link parameter 0.770

RESOLUTION LEVEL 2* Average link parameter 0.747
 Single link parameter 0.759

Cluster No.	Organism	
1	<u>S. aureus</u>	29
	<u>M. tetragenus</u>	
	<u>S. albus</u>	12731
2	<u>S. aureus</u>	42D
	<u>S. aureus</u>	2046
	<u>M. halophilus</u>	
	<u>S. aureus</u>	2057
3	<u>S. aureus</u>	2031
	<u>S. aureus</u>	2057
	<u>S. aureus</u>	42D
Satellite of Cluster 1	<u>S. albus</u>	12731
4	<u>M. luteus</u>	CCM 248
	<u>M. conglomeratus</u>	NCIB 2677
	<u>M. lysodeikticus</u>	NCIB 9278
Satellite of Cluster 4	<u>M. lysodeikticus</u>	NCIB 9278
5	<u>S. aureus</u>	3A
	<u>M. roseus</u> NCIB	8175
	<u>S. aureus</u>	2031
Satellite of Cluster 1	<u>S. aureus</u>	6
Organism not placed in Cluster	<u>M. sodonensis</u>	NCIB 8854

* Tables corresponding to resolution levels 1
and 2 are exactly similar.

TABLE XXIII

RESULTS FROM COMPUTER ANALYSIS OF GAS CHROMATOGRAMS
OF WHOLE CELLS GROWN ON NUTRIENT BROTH

RESOLUTION LEVEL 3 Average link parameter 0.725
 Single link parameter 0.747

RESOLUTION LEVEL 4* Average link parameter 0.702
 Single link parameter 0.736

RESOLUTION LEVEL 5* Average link parameter 0.680
 Single link parameter 0.725

Cluster No.	Organisms	
1	<u>S. aureus</u>	29
	<u>M. tetragenus</u>	
	<u>S. albus</u>	12731
	<u>M. lysodeikticus</u>	NCIB 9278
2	<u>S. aureus</u>	42D
	<u>S. aureus</u>	2046
	<u>M. halophilus</u>	
	<u>S. aureus</u>	2057
3	<u>S. aureus</u>	2031
	<u>S. aureus</u>	2057
	<u>S. aureus</u>	42D
4	<u>M. luteus</u>	CCM 248
	<u>M. conglomeratus</u>	NCIB 2677
	<u>M. lysodeikticus</u>	NCIB 9278
Satellite of Cluster 4	<u>M. lysodeikticus</u>	NCIB 9278
5	<u>S. aureus</u>	3A
	<u>M. roseus</u>	NCIB 8175
	<u>S. aureus</u>	2031
Organism not placed in clusters	<u>M. sodonensis</u>	NCIB 8854

*Tables corresponding to resolution levels 3, 4,
and 5 are exactly similar.

TABLE XXIV

RESULTS FROM COMPUTER ANALYSIS OF GAS CHROMATOGRAMS
OF WHOLE CELLS GROWN ON NUTRIENT BROTHRESOLUTION LEVEL 6 Average link parameter 0.657
Single link parameter 0.714

Cluster No.	Organism
1	<u>S. aureus</u> 29 <u>M. tetragenus</u> <u>S. albus</u> 12731 <u>M. lysodeikticus</u> NCIB 9278 <u>M. luteus</u> CCM 248
2	<u>S. aureus</u> 42D <u>S. aureus</u> 2046 <u>M. halophilus</u> <u>S. aureus</u> 2057
3	<u>S. aureus</u> 2031 <u>S. aureus</u> 2057 <u>S. aureus</u> 42D
4	<u>M. luteus</u> CCM 248 <u>M. conglomeratus</u> NCIB 2677 <u>M. lysodeikticus</u> NCIB 9278
5	<u>S. aureus</u> 3A <u>M. roseus</u> NCIB 8178 <u>S. aureus</u> 2031
Satellite of Cluster 1	<u>S. aureus</u> 6
Organism not placed in clusters	<u>M. sodonensis</u> NCIB 8854

TABLE XXV

RESULTS FROM COMPUTER ANALYSIS OF GAS CHROMATOGRAMS OF
WHOLE CELLS GROWN ON NUTRIENT BROTH

RESOLUTION LEVEL 7 Average link parameter 0.635

Single link parameter 0.702

Cluster No.	Organisms	
1	<u>S. aureus</u>	29
	<u>M. tetragenus</u>	
	<u>S. albus</u>	12731
	<u>M. lysodeikticus</u>	NCIB 9278
	<u>M. luteus</u>	CCM 248
2	<u>S. aureus</u>	420
	<u>S. aureus</u>	2046
	<u>M. halophilus</u>	
	<u>S. aureus</u>	2057
	<u>S. aureus</u>	2031
	<u>S. aureus</u>	29
3	<u>M. luteus</u>	CCM 248
	<u>M. conglomeratus</u>	NCIB 2677
	<u>M. lysodeikticus</u>	NCIB 9278
4	<u>S. aureus</u>	3A
	<u>M. roseus</u>	NCIB 8175
	<u>S. aureus</u>	2031
Satellite of Cluster 1	<u>S. aureus</u>	6
Organism not placed in clusters	<u>M. sodonensis</u>	NCIB 8854

TABLE XXVI

RESULTS FROM COMPUTER ANALYSIS OF GAS CHROMATOGRAMS OF
WHOLE CELLS GROWN ON NUTRIENT BROTH

RESOLUTION LEVEL 8	Average link parameter 0.590
	Single link parameter 0.680
RESOLUTION LEVEL 9*	Average link parameter 0.590
	Single link parameter 0.680
RESOLUTION LEVEL 10*	Average link parameter 0.567
	Single link parameter 0.669
RESOLUTION LEVEL 11*	Average link parameter 0.545
	Single link parameter 0.657
RESOLUTION LEVEL 12*	Average link parameter 0.552
	Single link parameter 0.646

Cluster No.	Organisms
1	<u>S. aureus</u> 29 <u>M. tetragenus</u> <u>S. albus</u> 12731 <u>M. lysodeikticus</u> NCIB 9278 <u>M. luteus</u> CCM 248 <u>M. conglomeratus</u> NCIB 2677 <u>S. aureus</u> 6 <u>S. aureus</u> 2057 <u>S. aureus</u> 2031 <u>S. aureus</u> 42D <u>S. aureus</u> 2046 <u>M. halophilus</u> <u>S. aureus</u> 3A
2	<u>S. aureus</u> 3A <u>M. roseus</u> NCIB 8175 <u>S. aureus</u> 2031
Organism not placed in cluster <u>M. sodonensis</u> NCIB 8854	

* Tables corresponding to resolution levels 8, 9, 10, 11 and 12 are exactly similar.

TABLE XXVII

RESULTS FROM COMPUTER ANALYSIS OF GAS CHROMATOGRAMS
OF NUCLEIC ACIDSRESOLUTION LEVEL 1 Average link parameter 0.770
Single link parameter 0.770

Cluster No.	Organisms		
1	<u>S. aureus</u>		2057
	<u>M. sodonensis</u>	NCIB	8854
	<u>M. halophilus</u>		
	<u>S. albus</u>		12731
	<u>S. aureus</u>		42D
	<u>S. aureus</u>		2031
2	<u>S. aureus</u>		187
	<u>S. aureus</u>		29
	<u>S. aureus</u>		2051
Satellite of Cluster 1	<u>S. aureus</u>		2051
Satellite of Cluster 1	<u>S. aureus</u>		2031
Satellite of Cluster 1	<u>M. conglomeratus</u>		2677
Satellite of Cluster 1	<u>S. aureus</u>		187B
Satellite of Cluster 2	<u>S. aureus</u>		2051
3	<u>S. aureus</u>		3A
	<u>S. aureus</u>		2046
	<u>M. sodonensis</u>	NCIB	8854
Organism not placed in clusters	<u>M. luteus</u>	CCM	248

TABLE XXVIII

RESULTS FROM COMPUTER ANALYSIS OF GAS CHROMATOGRAMS
OF NUCLEIC ACIDSRESOLUTION LEVEL 12 Average link parameter 0.550
Single link parameter 0.660

Cluster No.	Organisms	
1	<u>S. aureus</u>	2057
	<u>M. sodonensis</u>	NCIB 8854
	<u>M. halophilus</u>	
	<u>S. albus</u>	12731
	<u>S. aureus</u>	42D
	<u>M. luteus</u>	CCM 559
	<u>S. aureus</u>	2031
	<u>M. conglomeratus</u>	2677
	<u>S. aureus</u>	187B
	<u>S. aureus</u>	187
	<u>S. aureus</u>	29
	<u>S. aureus</u>	2051
	<u>M. luteus</u>	CCM 248
	<u>S. aureus</u>	2046
2	<u>S. aureus</u>	3A
	<u>S. aureus</u>	2046
	<u>M. sodonensis</u>	NCIB 8854

TABLE XXIX

RESULTS FROM COMPUTER ANALYSIS OF GAS
CHROMATOGRAMS OF PROTOPLASMS

RESOLUTION LEVEL 1 Average link parameter 0.770
Single link parameter 0.770

Cluster No.	Organisms	
1	<u>S. aureus</u>	2031
	<u>S. aureus</u>	2057
	<u>S. albus</u>	12731
2	<u>S. aureus</u>	6
	<u>S. aureus</u>	29
	<u>M. luteus</u> CCM	248
	<u>M. lysodeikticus</u>	NCIB 9278
Satellite of Cluster 1	<u>S. albus</u>	12731
Satellite of Cluster 2	<u>M. lysodeikticus</u>	NCIB 9278
Satellite of Cluster 1	<u>M. conglomeratus</u>	NCIB 2677
3	<u>S. aureus</u>	2057
	<u>M. halophilus</u>	
	<u>S. aureus</u>	187
Satellite of Cluster 1	<u>S. aureus</u>	187
Satellite of Cluster 2	<u>S. aureus</u>	42D
Organisms not placed in clusters	<u>M. tetragenus</u>	
	<u>M. luteus</u> CCM	248
	<u>S. aureus</u>	2046

DISCUSSION

Gas chromatography has only recently been considered as a possible method for assisting with the classification of bacteria. It is based on the principle that the mobile phase passes through the stationary phase and each component of the sample in the mobile advances at its own characteristic rate. In gas chromatography, the mobile phase is called the carrier gas (inert gas), and the stationary phase may be either a solid or, more commonly, a liquid with high boiling point on a finely divided solid packed into a column. The carrier gas is used to transport sample components from the injection point to the detector. Different components pass through the column at different speeds depending on their affinity towards the adsorbing material, therefore each component appears separately at the column outlet. The detector responds to the composition of the carrier gas stream as it leaves the column. The record of detector response as a function of time forms a chromatogram. The taxonomic differences as revealed by computer analysis of these chromatograms, and the limitations of the technique, are discussed in the General Discussion.

IV

GENERAL DISCUSSION

GENERAL DISCUSSION

Detailed study of 62 strains of Staphylococci and Micrococci using 31 biochemical tests revealed that all strains designated as Staphylococci (Staph. aureus and Staph. albus) were positive in conversion of nitrate to nitrite, casein hydrolysis, lipolysis, hippurate hydrolysis, mannitol fermentation and MRV-P test. All of them produced catalase and were tolerant to 5%, 7%, and 10% sodium chloride and 0.02% azide. All the strains of Staph. aureus with one exception, i.e. Staph. aureus 2046, produced DNase, coagulase and phosphatase. In contrast, all the Staph. albus strains were negative when tested for production of these three enzymes.

Biochemical tests of Micrococci revealed many different combinations of characters. With the exception of M. halophilus, none of the Micrococcus strains produced penicillinase. All the strains, except M. freudenreichii, produced catalase, and all oxidized glucose. The important thing indicated by the results is that there are often no well defined lines of demarcation between the so-called species in this group. Some of the strains resemble 'classical' Micrococci in some features and Staphylococci in other features. On the basis of simple inspection of results of biochemical tests it is impossible to make any valid statement regarding the taxonomic position of these transitional forms. Gibson (29) suggested that Staphylococci and Micrococci are connected by a series of intermediate forms.

However, the analysis of biochemical results with the aid of a computer which permits computation of measures of similarity indicated

that numerical taxonomy might be of value in spite of the availability in the present study of only 31 suitable characteristics. Ideally at least 50 characters should be chosen, but it is better to use a smaller number of valid characters than a larger series in which some of the characters may not be independent, or may depend upon tests of uncertain reproducibility. For example, some of the Micrococcus and Staphylococcus strains were tested for oxidation and fermentation of different sugars such as xylose, arabinose, lactose and mannitol. Most of the organisms failed to oxidize and ferment these sugars or gave very weakly positive results. Such results are difficult to score in a satisfactory way. The 31 characters chosen after extensive literature review were considered to be valid for the purpose of classification and to be sufficiently dependable for general use. Evans (23) also suggested the use of numerical taxonomy to describe the natural species in Micrococcaceae. However, he stressed the use of a large number of representative strains, using a wide array of carefully standardized tests. The need to standardize these tests has been emphasized by many workers, but it is a problem in itself, which requires much further work.

Cytochrome analysis of these organisms revealed a correlation between the pattern of cytochromes, oxidation and fermentation of glucose and results of the oxidase test. All the Staph. aureus and Staph. albus strains had cyt b + cyt a pattern, and oxidized and fermented glucose. They gave a negative oxidase test. In contrast, most of the Micrococci had cyt b, c, a, pattern of cytochromes. These organisms gave a positive

oxidase test and attacked glucose oxidatively but not fermentatively. With the exception of M. cyaneus strains NCIB 9148 and M. cyaneus CCM 856, and M. aurantiacus NCIB 8609, which have a Staphylococcus pattern of cytochromes (cyt b + cyt a) but attacked glucose oxidatively and not fermentatively, the above mentioned relationship between cytochromes and O/F and oxidase test existed in all the organisms tested. Five strains designated as Micrococci, i.e. M. halophilus, M. candidus NCIB 8610, M. tetragenus, M. freudenreichii NCIB 2699, and M. violagabriellae (Strains Parent, 2a, SP, LP) had the Staphylococcus pattern of cytochromes and likewise fermented and oxidized glucose and were oxidase negative. These results indicate that out of 62 strains of Staphylococci and Micrococci studied, 59 had complete correlation between the presence of cytochrome c and a positive result in the oxidase test. This is similar to the situation observed in 52 strains of Gram negative bacilli, among which two of the Pseudomonas strains 11319 pyo and 11319 pky were oxidase positive, attacked glucose oxidatively and possessed cyt c, whereas the other 50 Gram negative bacilli possessed a cytochrome b + cyt a pattern, but no cytochrome c, were oxidase negative, and attacked glucose oxidatively and fermentatively.

At this stage one can say that the family Coccaceae is characterized by 'typical' Staphylococci and 'typical' Micrococci, and intermediate or transitional organisms which resemble type strains of Staphylococci in some features and classical Micrococci in others. The strains which fall in the third category are: M. halophilus, M. tetragenus, M. candidus NCIB 8610, M. freudenreichii NCIB 2699, M. aurantiacus NCIB 8609,

M. cyaneus NCIB 9148 and M. cyaneus CCM 856.

This poses a problem of placing these organisms with the well defined Staphylococci or Micrococci or with neither. One requires some unbiased measure of similarity on the basis of which these organisms can be put in their respective places. Numerical taxonomy can make use of measures of similarity to find clusters of organisms. The program developed by George et al. (28), which has been described earlier, was used for this purpose. Analysis was done at 12 different levels of resolution starting from the highest - level 1, to the lowest, level 12. At high levels of resolution, where only the most closely related organisms are clustered together, these intermediate forms, along with some other organisms, are not allowed to join the major clusters of Staphylococci or Micrococci. At lower levels of resolution, M. halophilus, M. tetragenus, M. candidus NCIB 8610, M. freudenreichii NCIB 2699, which are similar to Staphylococcus in cyt pattern, O/F tests and oxidase tests, are grouped with Staphylococci. M. violagabriellae (Parent strains, 2a, LP and SP), obtained by Payne and Campbell (51) on UV irradiation of the parent strain, were clustered with Staphylococci at high levels of resolution, as they possessed the above mentioned characteristics of Staphylococcus, along with many other characteristics of this group. M. cyaneus NCIB 9148 and M. cyaneus CCM 856, M. aurantiacus NCIB 8609, which resemble Staphylococcus in cytochrome pattern and Micrococcus in O/F tests, are placed neither with the staphylococcal nor with the micrococcal cluster of organisms. Even at the lowest level of resolution (level 12), which makes use of

low similarity values, these organisms are not allowed to join any cluster. This can be seen in Tables IV - IX.

These findings are consistent with those of Auletta (3), who studied the base composition of 36 strains of Staphylococcus and Micrococcus, including M. candidus, M. violagabriellae, and M. freudenreichii. These three organisms had a GC (guanine + cytosine) content of 30%, which is in the range found for 'typical' staphylococcal strains (GC 30 - 39%). Steel (66) has also emphasized that biochemically and culturally, M. candidus and M. violagabriellae correspond with the description of Staphylococci by the criteria of Baird-Parker (4) and Staph. saprophiticus by the criteria of Shaw et al. (57). M. freudenreichii had a GC content of 59% which is relatively low as compared with most of the Micrococci, which had a GC content of 65 - 72%, and high compared with the GC content of most of the Staphylococci. Gause et al. (27) reported that after UV irradiation of Staphylococcus with a DNA base composition of 32.4% GC content, several small colony mutants were isolated whose DNA base composition varied between 69.2% and 71% GC. These % GC values for these mutants are typical of Micrococci. Silvestri (58) regards these intermediate organisms as evolutionary links between Staphylococcus and Micrococcus.

Apart from the finding of true natural clusters on the basis of a large number of tests, the above program can also be used to examine the clusters obtained by submitting a range of strains to a smaller selected series of tests and the placing of particular strains in relation to the main clusters detected. This is what has been done here, and this procedure permits a retrospective scan of the tests to facilitate

selection of smaller sets of tests which might have differential value in identification. The procedure of numerical taxonomy indicates that at high levels of resolution, many of the organisms form clusters, each of which contains only organisms having the same conventional generic and specific names. At resolution level 1 (Table IV) there are five clusters of designated *Staphylococcus* strains (clusters 4, 5, 6, 8, 9). Clusters 4 (phage typable), 5 and 8, include all the strains of *Staph. aureus*. Organisms designated *Staph. albus* are grouped into two clusters (clusters 6 and 9). At resolution level 2 (Table V) all the above three clusters of *Staph. aureus* constitute one cluster, namely cluster 4, and all the *Staph. albus* strains (with the exception of one strain) are put into cluster 5. All the *Micrococci* (with the exception of a few strains) fall into clusters 1, 2, 3, 6. *M. varians* CCM 1046, *M. flavus* NCIB 8166 form a cluster of their own, i.e. cluster 7. *M. lysodeikticus* NCIB 9278 and a few *M. luteus* strains in this cluster are indicated as satellites of clusters 1, 2. *M. violagabriellae* strains (except LP) are in cluster 6.

Gas chromatography proved to be a potentially useful technique for the analysis of whole cells of *Staphylococci* and *Micrococci* grown on nutrient agar or in nutrient broth. Since the differences between these organisms are small, only high levels of resolution can detect these differences and hence indicate a possibly valid classification. With successively lower levels of resolution there is a decrease in the coefficient of similarity necessary for cluster membership and hence a drop in sensitivity to small differences. Therefore, for the

purpose of classification of Staphylococci and Micrococci, resolution levels 1 and 2 are considered to be of value. The organisms are placed mainly into two types of clusters. One type of cluster includes organisms of the Staphylococcus group and the other type those of the Micrococcus group. Organisms like M. halophilus; M. tetragenus, M. candidus, which are clustered with the Staphylococcus group on analysis of physiological and biochemical analysis, also cluster with the same group on analysis of their chromatograms. Micrococcus roseus NCIB 8175, which enters the biochemical cluster 1 (Micrococci) only at level 12, was placed with Staphylococci on the basis of its gas chromatogram. This organism needs further study. There are marked differences in the chromatograms of Staphylococci and Micrococci, and these differences are in peak ratios rather than the absence or presence of certain peaks. Curves for comparison are shown in Figures 6 - 19. The peaks in the range of 14 - 20 for whole cells of Staphylococci and Micrococci grown on nutrient broth differ markedly from each other. The pattern of topography of peak nos. 18, 19 and 20 in Micrococci is in the ascending order, whereas in Staphylococci, i.e. in Staph. aureus as well as in Staph. albus organisms, peaks nos. 18 and 19 are in ascending order, whereas peaks 19 and 20 are in descending order. Since the area under each peak is proportional to the concentration of the corresponding component in the mixture, the difference in the ratio of the peaks (e.g. 18, 19 20) of whole cells of Staphylococci and Micrococci are due to the different amounts of compounds derived from pyrolysis of whole cells. Gas chromatographic analysis of different pyrolysis products of

individual components such as nucleic acids and protoplasts of these organisms did not give clusters similar to those obtained with whole cells. It is clear from Table XXVII (Level 1 of nucleic acids) that even at the highest level of resolution there are no corresponding clusters. For example, cluster 1 formed under resolution level 1 includes Staph. aureus 2057, Staph. aureus 42D, Staph. aureus 2031, M. sodonensis NCIB 8854, M. halophilus, Staph. albus 12731. It can be seen from the gas chromatograms of nucleic acids (Figs. 14, 15) that there are certain differences, but it was found that in successive runs the differences were not consistent, suggesting technical limitations in this approach. The same applies to the gas chromatograms of protoplasts of different organisms.

It is possible that gas chromatography of hydrolysis products of DNA and RNA, by methods other than pyrolysis, would be of greater value. The organisms do differ in their physiological reactions such as production of DNase, coagulase and phosphatase. The production of these enzymes is directed by the nucleic acids, and one gene might direct the synthesis of an enzyme. Differences in the physiology of these organisms are due to differences in nucleic acids, but a small difference in nucleic acids may lead to a big difference in the phenotype of the physiology of the organism. To detect the presence, absence, or modification of one gene is beyond the scope of pyrolysis gas chromatography. Other characteristics of nucleic acids such as base ratios studied by Hill (32) and base composition studied by

Schildkraut (56) and Auletta (3) are better criteria for classification. Analysis of nucleic acids or protoplasms with the aid of pyrolysis gas chromatography was of no obvious taxonomic value, and the results obtained clearly indicate some of the limitations of the technique. Certain difficulties are encountered in the practical application of gas chromatography. The selection of operating conditions such as the nature of the stationary phase and the mobile phase, length of the column, temperature range of the program, heating rate and gas flow rate, and the temperature of pyrolysis are very important. The reproducibility of these conditions from one run to another is not always satisfactory. The heating rate and the gas flow can be adjusted but the nature of the stationary phase, which changes with successive runs, cannot be brought back to exactly the original state. The change in the state of the column is brought about by the accumulation of impurities and the 'bleeding' of the liquid from the stationary phase in the column.

It was noted during the course of this study that not only the operating conditions, but the manner in which two samples of the same organism are prepared may also have a marked influence on the chromatograms. For example, the cells grown on nutrient agar gave chromatograms which differed markedly from those of cells grown in nutrient broth. This can be seen from the chromatograms of whole cells of Staph. albus 12731 and M. halophilus grown on two different kinds of media (nutrient broth and nutrient agar). Not only different kinds of media, but the utilization of different brands of the same medium led to a considerable variability in the chromatograms. The effect of different kinds of media

on the variability of chromatograms is shown in Figures VI, VII, VIII, IX. Since this was noticed in the early phases of experimentation, the media for further use were prepared in large batches to accommodate the growth of all the organisms required for a particular analysis.

All the findings reported here support the view that a third group might legitimately be defined within the family Coccaceae, along with the two existing groups of Staphylococcus and Micrococcus. It is not possible, because of the small number of strains available for this study, to give any estimate of the degree of heterogeneity of this group.

It is possible to view the Staphylococcus-Micrococcus complex as a spectrum of organisms within the family Coccaceae, with the 'classical' Micrococci at one end and the 'classical' Staphylococci at the other, with intermediate forms in between. These forms consist of organisms frequently designated as Micrococci. One set of these organisms, i.e. M. halophilus, M. tetragenus, M. candidus NCIB 8610, M. freudenreichii NCIB 2699, fits best with Staphylococci on the basis of biochemical tests, cytochrome patterns and numerical analysis of gas chromatograms of whole cells grown on nutrient agar and nutrient broth. The other set of organisms (M. cyaneus NCIB 9148, M. cyaneus CCM 856 and M. aurantiacus NCIB 8609) is placed neither with Staphylococci nor with Micrococci even at low levels of resolution. This classification requires further validation because the number of available strains of these intermediate forms designated as Micrococci was small. The present study does demonstrate the principles of one meaningful approach to a complex problem, and two suggestions based on the results and observations could be of value to microbiologists who need to identify strains of Gram positive

cocci which appear to belong to the Staphylococcus-Micrococcus complex.

Firstly, a small number of selected tests such as the tube-oxidase test, cytochrome pattern and O/F reactions should be used. These tests seem to correlate well with a useful division of strains. For example, those organisms which are oxidase negative, attack glucose oxidatively and fermentatively and possess cyt b and cyt a, may provisionally be called Staphylococci and those which are tube-oxidase positive, attack glucose only oxidatively, and possess cyt b, cyt c and cyt a, may be called Micrococci.

Secondly, the classification based on clusters computed from a wide array of characters of a large number of strains should be worked out. The test results for all the strains could be stored in the form of a matrix in a computer program. When faced with an unknown organism to be identified, a series of tests could be performed and the computer used to match the unknown to the stored test results. This would indicate the strain or strains in the stock set to which the unknown is most closely related. It would also be possible to re-run the cluster program, adding each strain to be identified, which simply involves the addition of one more data card containing the test results of the organism to be identified to the pre-existing data cards of other organisms. These cards, along with the main program cards, when fed into the computer, will provide information for a new cluster analysis. This procedure is more suitable for exact placing of the organisms, but for practical purposes is more laborious. The use of a simple matching procedure with reference back to results determined on the basis of a wide selection of strains would be more convenient in everyday use.

The continued application of methods of this kind would help to put microbial identification on a sound, quantitative basis, and would lead to the rapid accumulation of a large amount of useful information. The necessary standardization of methods would facilitate the comparison of results obtained in different laboratories where the system was introduced.

The gas chromatographic results suggest that the clusters obtained by this means alone would differ in details of cluster membership from those defined on the basis of biochemical tests. A combination of the two approaches is possible, although certain difficulties might arise in determination of methods for coding the data so that they could be included in the computer analysis. On general grounds, it seems likely that no one approach will displace others in defining characters for taxonomic studies and that the results from a number of different analyses could be combined. These might include data on: morphology and staining; ultrastructure; gross colonial form and color under carefully controlled conditions, preferably on defined media; conventional biochemical tests; components detected by acrylamide gel electrophoresis; features of gas chromatograms obtained by gas chromatography, and other methods; Infra-red spectrograms.

In the application of gas chromatography to these problems, a difficulty which might have to be considered is that the large degree of overall similarity between pyrolysis chromatograms of different bacteria might mask small but highly significant differences. The addition of a gas chromatographic study of metabolic products formed under strictly

controlled conditions from selected substrates might add considerably to the usefulness of this technique.

The necessary computer facilities and analytical equipment necessary for the type of work described in this thesis are becoming more readily available to diagnostic services than they were in the past. Suitable APL programs for use as diagnostic aids have been developed in the Department of Bacteriology (Jackson, F. L. - unpublished). For this reason, experimental study of possible fields of application, leading to determination of the strengths and weaknesses of the newer methods would appear to be fully justified.

SUMMARY AND CONCLUSIONS

SUMMARY

Sixty-two strains of organisms loosely designated as Staphylococci and Micrococci were classified on the basis of biochemical tests, cytochrome patterns and pyrolysis, gas chromatography of whole cells grown on nutrient agar and nutrient broth. Different components (nucleic acids, protoplasm, cell walls) of these organisms were separated and analyzed by pyrolysis gas chromatography. In addition to the simple inspection of the results, the data were also analyzed with a cluster analysis computer program.

The organisms designated as Staphylococci were characterized by cyt b, cyt a pattern of cytochromes and utilized glucose oxidatively and fermentatively. They were positive in conversion of nitrite to nitrate, casein hydrolysis, lipolysis, hippurate hydrolysis, mannitol fermentation and MR test and oxidase test. All produced catalase and were tolerant to 10% sodium chloride and 0.02% sodium azide. With the exception of one strain, i.e. Staph. aureus 2046, all the Staph. aureus strains produced DNase, coagulase, phosphatase. All the Staph. albus strains were negative in the production of these three enzymes.

Most of the Micrococci had cyt b, cyt c, cyt a pattern of cytochromes. A few of the organisms designated Micrococci, M. halophilus, M. tetragenus, M. candidus NCIB 8610, M. freudenreichii NCIB 2699, M. cyaneus NCIB 9148, M. cyaneus CCM 856, and M. aurantiacus, had staphylococcal patterns of cytochromes (cyt b, cyt a). Likewise, (with the exception of M. cyaneus NCIB 9148, M. cyaneus CCM 856, M. aurantiacus NCIB 8609) all the above-mentioned organisms gave a positive

oxidase test and attacked glucose oxidatively and fermentatively, whereas most of the organisms with typical micrococcal type of cytochrome pattern attacked glucose only oxidatively and gave a positive oxidase test.

Computer analysis of the biochemical tests and cytochrome patterns, using a cluster analysis program based on overall similarity, gave meaningful clusters. At high levels of resolution all the Staph. aureus strains were separated from the Staph. albus strains, and the Micrococci were put into separate clusters, except M. violagabriellae strains (Parent, 2a, SP, LP), which have staphylococcal pattern of cytochromes and resemble the Staphylococci in many of the biochemical tests. These were included with Staphylococci at high levels of resolution. Other organisms designated Micrococci but possessing characteristics of both the major groups, i.e. Micrococci and Staphylococci, were placed with Staphylococci at fairly high levels of resolution and M. cyaneus NCIB 9148, M. cyaneus CCM 856, and M. aurantiacus, which could be placed neither with Staphylococci nor Micrococci were excluded even at the lowest level of resolution. Essentially similar results were obtained in a pilot study of analysis of gas chromatograms of whole cells of selected strains grown on NYA and NYB media. Computer analysis of these results gave meaningful clusters of organisms. The results not only add suggestions for the taxonomic position of some of the transitional forms, but also indicate that visual spectroscopy is valuable as an aid to classification of Staphylococci and Micrococci and that gas chromatography might have

considerable potentiality for detection of features suitable for taxonomic studies.

CONCLUSIONS

The oxidase reaction is a useful feature of the strains examined. The tube test developed for this study can be standardized and gives reproducible results.

Pyrolysis gas chromatography has potentialities for comparison of whole cells grown under carefully controlled conditions. Typical Staphylococci and Micrococci were separated by analysis of their chromatograms. Clusters detected on the basis of chromatograms differed in details of membership from those based on biochemical reactions, but in each series the 'Staphylococcus-like' Micrococci were placed with Staphylococci. A discrepancy was found for M. roseus NCIB 8175, which was placed with Staphylococci on the basis of its chromatogram. This organism enters a biochemical micrococcal cluster only at level 12, and requires further study. Gas chromatograms of cell fractions (protoplasm devoid of cell wall, nucleic acids) were not of value for this study. Visual inspection of chromatograms may draw attention to differences between closely related organisms.

Visual spectroscopy is technically simple, and the cytochrome patterns appear to be of considerable value for provisional classification, if combined with a few other features.

The results suggest that among the organisms examined, four major varieties can be distinguished. These are:

1. The 'typical' Micrococci. These are
 - (a) oxidase positive
 - (b) contain cyt b, cyt c, cyt a, and

(c) attack glucose oxidatively.

2. The 'typical' Staphylococci. These are

(a) oxidase negative

(b) contain cyt b and cyt a, and

(c) attack glucose fermentatively.

3. Certain organisms hitherto designated Micrococci, but probably fitting better into the Staphylococcus clusters, and placed with Staphylococci on the basis of the tests used in this study.

These are:

<u>M. freudenreichii</u>	NCIB 2699
<u>M. halophilus</u>	
<u>M. candidus</u>	NCIB 8610
<u>M. tetragenus</u>	
<u>M. violagabriellae</u>	(Strains
	Parent, 2a, Super pig, Little pig)

They are

(a) oxidase negative

(b) contain cyt b and cyt a components

(c) attack glucose fermentatively.

4. Odd strains not placed in clusters, even at level 12, on the basis of the tests used. These are:

<u>M. cyaneus</u>	NCIB 9148
<u>M. cyaneus</u>	CCM 856
<u>M. aurantiacus</u>	NCIB 8609

These are:

(a) oxidase negative

(b) contain cyt b, cyt a

(c) attack glucose oxidatively.

VI

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